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Silver-Russell Syndrome and Human Growth: Genetic and Epigenetic Studies

Mari Muurinen

ACADEMIC DISSERTATION

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ABBREVIATIONS

11p15 LOM	loss of methylation at chromosome 11p15
3-M syndrome	a syndrome named after researchers Miller, McKusick and Malvaux
AGA	appropriate for gestational age
AGPAT5	1-acylglycerol-3-phosphate O-acyltransferase 5
AS	Angelman syndrome
BMI	body mass index
BWS	Beckwith-Wiedemann syndrome
CARD11	caspase recruitment domain family member 11
cDNA	complementary DNA
CDKN1C	cyclin dependent kinase inhibitor 1C
CF	cystic fibrosis
CGI	CpG island
CNTNAP2	contactin associated protein like 2
CNV	copy number variation
COL1A2	collagen type I alpha 2 chain
CpG	a site in the DNA sequence where a cytosine (C) is followed by a guanine (G) nucleotide
CSRP1	cysteine and glycine rich protein 1
CTCF	CCCTC-binding factor
Da	Dalton
DMR	differentially methylated region
DNA	deoxyribonucleic acid
DNAse I	deoxyribonuclease I
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
dNTP	deoxyribonucleotide triphosphate
EZH2	Enhancer of Zeste Homolog 2
FRMD6	FERM domain containing 6
GH	growth hormone
GLI3	GLI family zinc finger 3
GNAS	guanine nucleotide binding protein alpha stimulating complex locus
GPC5	glypican 5
GPC6	glypican 6
GR	glucocorticoid receptor
GRB10	growth factor receptor bound protein 10
GWAS	genome-wide association study
H19	*H19*, imprinted maternally expressed transcript
H19/IGF2:IG-DMR	*H19-IGF2* intergenic differentially methylated region
HIL	hypomethylation of multiple imprinted loci
HOX genes	a subset of homeobox-containing genes that regulate the anterior-posterior patterning of an embryo
HOXA4	homeobox A4
HTR5A	5-hydroxytryptamine (serotonin) receptor 5A
IC	imprinting center
ICR	imprinting control region
IGB	integrated genome browser

IGF1	insulin-like growth factor 1
IGF1R	insulin-like growth factor 1 receptor
IGF2	insulin-like growth factor 2
Igf2r	insulin-like growth factor 2 receptor
IMAGe	intrauterine growth restriction, metaphyseal dysplasia, congenital adrenal hypoplasia and genital anomalies
ISS	idiopathic short stature
ITGB8	integrin subunit beta 8
IUGR	intrauterine growth restriction
Kcnq1	potassium voltage-gated channel, subfamily Q, member 1
Kcnq1ot1	*Kcnq1* overlapping transcript 1
KOS	Kagami-Ogata syndrome
lncRNA	long non-coding RNA
LOI	loss of imprinting
LOM	loss of methylation
MAD1L1	mitotic arrest deficient 1 like 1
MALDI-TOF MS	matrix assisted laser desorption/ionization – time of flight mass spectrometry
MBCS	Mulchandani-Bhoj-Conlin syndrome
MEST	mesoderm specific transcript
MESTIT1	MEST intronic transcript 1, antisense RNA
MHS	multilocus hypomethylation syndrome
MLH	multilocus hypomethylation
MLID	multi-locus imprinting disturbance
MLMD	multilocus methylation defects
mRNA	messenger RNA
Mulibrey nanism	MUscle, LIver, BRain, EYes nanism
ncRNA	non-coding RNA
NH-CSS	Netchine-Harbison clinical scoring system
PCR	polymerase chain reaction
PEG3	paternally expressed 3
PEG10	paternally expressed 10
PHLDA2	pleckstrin homology like domain family A member 2
PHP1B	pseudohypoparathyroidism type 1b
PLAGL1/HYMAI	PLAG1 like zinc finger 1/hydatidiform mole associated and imprinted
PON1	paraoxonase 1
POU6F1	POU class 6 homeobox 1
PWS	Prader-Willi syndrome
PRR15	proline rich 15
qPCR	quantitative real-time polymerase chain reaction
RARRES2	retinoic acid receptor responder 2
RERG	RAS like estrogen regulated growth inhibitor
RFLP	restriction fragment length polymorphism
RIN	RNA integrity number
RNA	ribonucleic acid
RPS2P32	ribosomal protein S2 pseudogene
SD	standard deviation
SGA	small for gestational age
SGR	severe growth restriction of unknown etiology
SGCE	sarcoglycan epsilon
SH2B2	SH2B adaptor protein 2
SH3MD4	SH3 domain containing ring finger 3

SHORT	short stature, hyperextensibility of joints, ocular depression, Rieger anomaly and teething delay
SHOX	short stature homeobox
SNP	single nucleotide polymorphism
SOX21	SRY-box 21
SPOCK1	SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1
SRS	Silver-Russell Syndrome
Sty I	a restriction enzyme purified from E. coli WA921/pST27 hsd+
SVOPL	SVOP like
TFCP2	transcription factor CP2
TFBS	transcription factor binding site
TNDM	transient neonatal diabetes mellitus
TS	Temple syndrome
TSS	transcription start site
ueSRS	unexplained Silver-Russell Syndrome
UPD	uniparental disomy
UPD(7)mat	maternal uniparental disomy of chromosome 7
UPD(7)pat	paternal uniparental disomy of chromosome 7
VLBW	very low birth weight
ZFP57	zinc finger protein 57

ABSTRACT

A large part of the genetic and epigenetic changes contributing to human height and growth remain unknown. Silver-Russell syndrome (SRS) is a rare human growth disorder, where maternal uniparental disomy of chromosome 7 [UPD(7)mat] and loss of methylation at 11p15 (11p15 LOM) are the two major findings. However, a substantial proportion of the SRS patients remain without a molecular diagnosis. SRS is caused by a disturbance of imprinted genes that are expressed solely or predominantly from one parent and are important for growth and development. Imprinted genes may also be especially susceptible to environmental factors, which can cause persistent changes in methylation patterns and affect development of adult-onset diseases.

This thesis aims to explore new genetic and epigenetic changes in SRS, as well as epigenetic changes of imprinted genes in growth-restricted children, children of normal growth and individuals born pre-term.

In Study I, genomic structural variation of 22 SRS patients was studied with the Affymetrix 250K Sty microarray. Several copy number changes were found, including a heterozygous deletion of 15q26.3 including the insulin-like growth factor 1 receptor (*IGF1R*) gene.

In Study II, CpGs in the insulin-like growth factor 2 (*IGF2*) gene (located in the 11p15 region) were tested in pre-term and very low birth weight born individuals with elevated levels of cardiovascular risk factors. DNA methylation changes were found in one CpG site of the pre-term born individuals compared to controls.

In Study III, genome wide comparisons between the DNA methylation levels of SRS patients that have UPD(7)mat, controls, and an individual with paternal uniparental disomy of chromosome 7 [UPD(7)pat] were done. DNA methylation was studied using the Illumina Infinium HumanMethylation450K BeadChip technology, which is capable of measuring methylation level of more than 450

000 CpG sites across the genome. The study provided new information on the DNA methylation landscape of chromosome 7, suggesting new differentially methylated regions (DMRs) and imprinted genes.

In Study IV, DNA methylation levels were compared between three different subgroups of SRS: SRS with UPD(7)mat, SRS with 11p15 LOM and clinical SRS without a known molecular etiology. The promoter region of homeobox A4 (*HOXA4*) gene was found hypomethylated in all of the subgroups of SRS. This region was subsequently tested in other severely growth-restricted patients and was found hypomethylated. Additionally, the methylation level of the *HOXA4* promoter region was found to be associated with the height of school-aged children, suggesting that *HOXA4* plays a role not only in SRS but also in the regulation of height in general.

These studies found molecular changes in SRS, new differentially methylated regions in chromosome 7 and epigenetic findings potentially relevant for regulation of human growth. The findings provide potential targets for further studies in human growth and development.

INTRODUCTION

Human height is a complex trait with high heritability. As demonstrated by large genome-wide association studies (GWAS), human height is regulated by hundreds of genes, with each locus typically explaining a small proportion of the variation. It has been debated to what extent these loci explain the heritability of human height, but many factors contributing to normal variation of human height still remain unknown.

Short stature, or poor growth in children, is a symptom that can be due to many factors including malnutrition, systemic disorders, or an underlying genetic or epigenetic defect. Genomic studies have located many monogenic disorders responsible for severe short stature. Finding the molecular cause for a growth disorder is important for planning appropriate care and genetic counseling.

SRS is a rare human growth disorder, in which both prenatal and postnatal growth is affected. SRS patients are born small for gestational age, they are shorter than their peers during childhood and reach an adult height below that of the usual height variation. SRS belongs to a group of disorders called imprinting disorders, which often affect growth and neurological functioning. While most human genes are expressed from both parents, imprinted genes are an exception to the rule – they are expressed solely or predominantly either from a person's mother or father, not both. DNA methylation, the addition of methyl groups to DNA, is the most important and easily assayed epigenetic mark involved in imprinting. In SRS, imprinted genes can be dysregulated either through genomic aberrations or epimutations, but for a large part of SRS patients the etiology remains unknown.

The studies in this thesis utilize multiple approaches, such as genome-wide arrays and targeted studies, to explore the genetic and epigenetic factors contributing to SRS. Those factors are then examined in other growth-restricted children without SRS, as well as in children of normal growth. Additionally,

epigenetic changes are studied in adults born pre-term at very low birth weight, in order to study epigenetic changes of the imprinted gene, *IGF2*.

REVIEW OF THE LITERATURE

The human genome

All human cells contain hereditary information in the form of deoxyribonucleic acid (DNA). Most DNA is located in the cell nucleus, while a small part is present in mitochondria. DNA consists of nucleotides, units containing a deoxyribose sugar, a phosphate group and one of four bases: adenine (A), thymine (T), guanine (G) and cytosine (C). DNA has a double-helix structure, in which the bases on opposite strands are connected by hydrogen bonds: A bonds with T, and C bonds with G.

Nuclear DNA is packaged in bead-like units called nucleosomes, which consist of two turns of genomic DNA wrapped around eight histones (two of each of the four types: H2A, H2B, H3 and H4). These nucleosomes make up chromatin that exists in two different forms: a loose decondensed form called euchromatin that allows for DNA regulatory processes to take place, and a condensed heterochromatin that generally lacks regulatory activity (Tollefsbol, 2011). Further folding and condensing of the chromatin during cell division makes a chromosome visible under a microscope.

Most human cells contain 22 pairs of autosomal chromosomes and one pair of sex chromosomes, XX for females and XY for males. One chromosome of each pair is inherited from the mother and one from the father. Altogether these chromosomes contain around 3 billion DNA base pairs and more than 20 000 protein-coding genes (Willyard, 2018). Mitochondrial DNA exists in circular molecules of 16 569 DNA base pairs that encode for 37 genes (Shokolenko & Alexeyev, 2015), and it is only inherited from the mother.

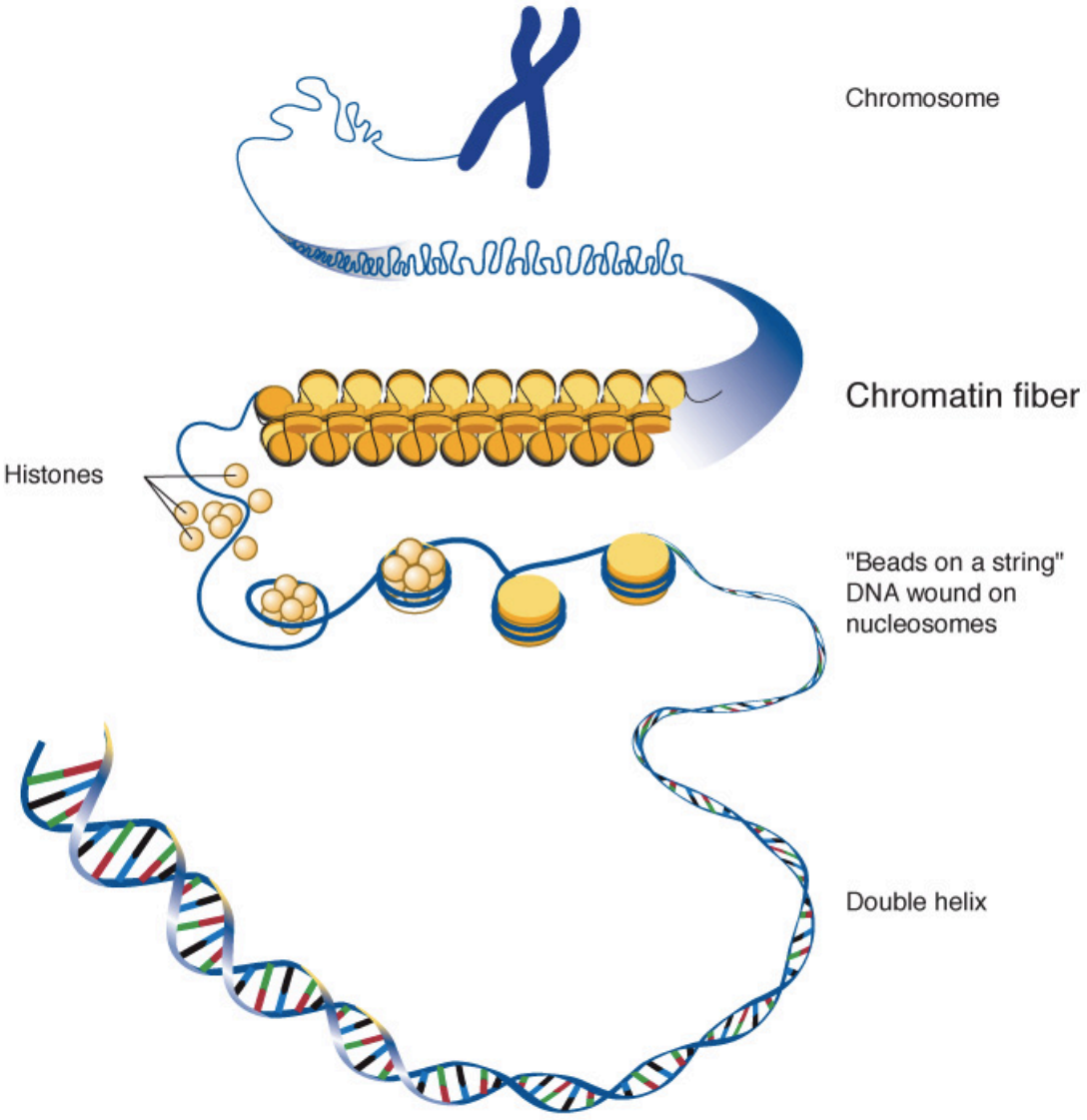


Figure 1. Packaging of DNA.

Courtesy: National Human Genome Research Institute (www.genome.gov)

The central dogma of molecular biology describes how genetic information from DNA is transcribed into ribonucleic acid (RNA), which is then translated into protein. Protein-coding genes contain exons, which are the protein-coding segments, and introns, which do not code for a protein. During transcription, both exons and introns are first transcribed to messenger RNA (mRNA). Introns are then removed by splicing and the final mRNA is translated into protein. Gene transcription is activated and regulated by transcription factors that bind to specific DNA sequences (transcription factor binding sites, TFBS) at gene promoters or enhancers (Lenhard et al., 2012). The accessibility of regulatory elements to transcription factors is affected by chromatin structures and transcription factors can gain access to regulatory elements by recruitment of chromatin-modifying activities (Voss & Hager, 2014). Many promoters colocalize with CpG islands, which are regions rich in CpG dinucleotides. Transcription factor binding can also be strongly influenced by DNA methylation (the addition of a methyl group to the cytosine of a CpG dinucleotide) at transcription factor binding sites (Jones, 2012).

Variation in the human genome

Variation in the human genome contributes to the differences between the phenotypes of individuals and can sometimes lead to disease. This variation ranges from changes of a single nucleotide to large changes involving parts of chromosomes or even entire chromosomes. Mutations of single nucleotides can occur by substitution, deletion or insertion of a nucleotide (Figure 2). Single nucleotide polymorphisms (SNPs) are a type of variation of a single nucleotide, occurring in the normal population with a frequency >1%. Larger mutations include copy number variations (CNVs), such as deletions and duplications, that result in gain or loss of genetic information (Figure 2). Copy number neutral variations of the genome include inversions, translocations and uniparental disomy (UPD).

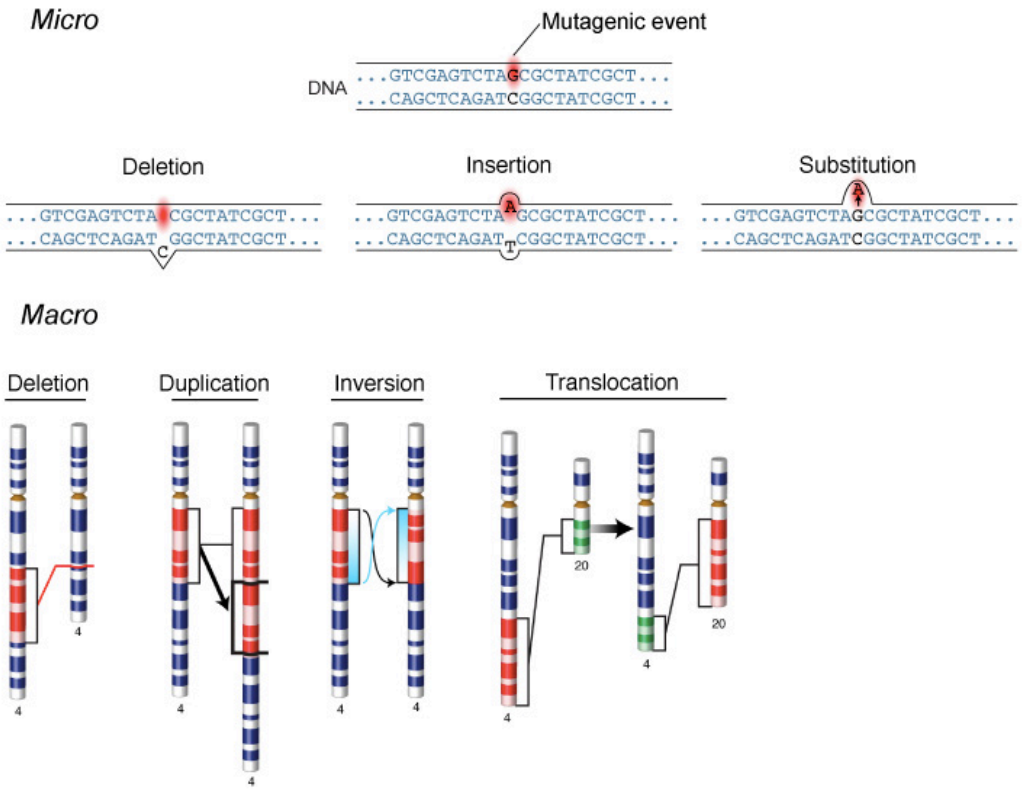
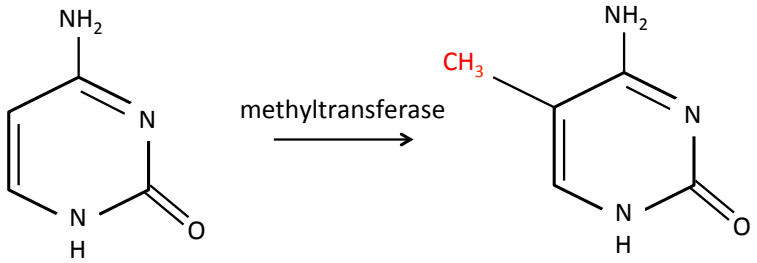


Figure 2. Mutations of variable sizes. Modified from www.genome.gov (National Human Genome Research Institute)

Epigenetics

The term epigenetics can be defined as "the collective heritable changes in phenotype due to processes that arise independent of primary DNA sequence" (Tollefsbol, 2011). The term "epi-" also means "on top of", which refers to changes that are additional to the DNA sequence. Epigenetic mechanisms, such as DNA methylation and histone modifications, cause changes in gene expression without altering the genome. DNA methylation refers to the addition of a methyl (CH_3) group to the 5-position of the cytosine of a CpG dinucleotide by methyltransferase enzymes (DNMTs). DNA methylation is heritable in cell division and it often results in down-regulation of gene activity when present in a gene regulatory region. Chromatin structure is influenced by post-translational

modifications to the histone proteins, such as acetylation or methylation. Increased histone acetylation is generally associated with increased gene activity, whereas histone methylation can be activating or repressing depending on the type of modification. Histone modifications also often coordinate with DNA methylation. (reviewed in Tollefsbol, 2011)



Cytosine 5-methylcytosine

Figure 3. DNA methylation

Epigenetics plays a role in a number of important processes, such as cellular differentiation, X chromosome inactivation and genomic imprinting. This thesis is largely focused on genomic imprinting and DNA methylation changes.

Genomic imprinting

Imprinted genes are a small group of genes that are solely or predominantly expressed from one parental chromosome. Through epigenetic mechanisms, one parental allele is silenced, while the other is expressed. This parent-of-origin dependent expression is essential for normal development. A disturbance that alters the normal dosage of imprinted genes can lead to abnormalities in growth and neurological functioning (Ishida & Moore, 2013). Loss of imprinting is also linked to the development of cancer (Feinberg et al., 2006).

Genomic imprinting was first discovered by studies that produced mouse embryos that contained either only maternal or paternal genomes (McGrath &

Solter, 1984) (Surani et al., 1984) (Barton et al., 1984). It was observed that the androgenetic or gynogenetic embryos did not complete normal embryogenesis (McGrath & Solter, 1984). The male-derived embryos developed poorly with relatively well-developed extraembryonic tissues, while the female-derived embryos had a better embryonic development with poor extraembryonic tissues (Barton et al., 1984)(Surani et al, 1984). These studies demonstrated that maternal and paternal genomes are not equal and that contribution from both parents is required for normal embryogenesis.

Studies of mice with uniparental disomies demonstrated that certain chromosomes, such as chromosome 11, contain imprinted regions. Mice with two maternal copies of chromosome 11 were consistently smaller, and mice with two paternal copies were consistently larger than their littermates (Cattanach & Kirk, 1985). Observations of the inequality of parental genomes lead to the parental conflict (kinship) theory, which proposed that imprinting evolved in mammals because of conflict of interest between the maternal and paternal genomes in relation to the transfer of nutrients from the mother to the offspring (Moore & Haig, 1991). According to the theory, paternally expressed imprinted genes would act to extract resources from the mother to achieve maximal fetal growth for the offspring, while maternally expressed imprinted genes would act in the opposite way to conserve maternal resources in order to enable possible future pregnancies.

Single imprinted genes were first identified in 1991 in mouse studies, when insulin-like growth factor 2 receptor (*Igf2r*) (Barlow et al., 1991), *Igf2* (DeChiara et al., 1991) and *H19,* imprinted maternally expressed transcript (*H19*) (Bartolomei et al., 1991), were described as imprinted. Currently there are a 100 known imprinted genes in human, more than a 100 predicted imprinted genes and additional genes with provisional or conflicting data about imprinting (Jirtle, Imprinted Gene Databases).

Imprinting involves various mechanisms, including DNA methylation, histone modifications, chromatin insulators and non-coding RNAs (Lewis & Reik, 2006). Most imprinted genes are located in clusters that are regulated by chromatin

insulators or long non-coding RNAs (lncRNAs) (Barlow & Bartolomei, 2014). DNA methylation has a central role in imprinting because the maternal and paternal alleles can be distinguished from one another when only one parental allele is methylated at differentially methylated regions (DMRs). Some DMRs function as imprinting centers (ICs), known also as imprinting control regions (ICRs) (Lewis & Reik, 2006). ICRs control the expression of the genes in the imprinting cluster in a parent-of-origin specific manner.

Parental imprints are reprogrammed at each generation to allow establishment of the imprints according to the sex of the contributing parent for the next generation. During reproduction, global epigenetic reprogramming occurs first in the germline and again at fertilization (reviewed in Mackay & Temple, 2017). The first wave of reprogramming affects also imprinted genes, so that imprints are erased in the germline and then re-set according to the parental origin. The parental imprints, however, are not affected by the second wave of reprogramming after fertilization. These imprints are mitotically stable and they are maintained throughout development of the organism (Horsthemke, 2010). DNA methyltransferases, including DNA methyltransferase 3A (DNMT3A) and DNA methyltransferase 3B (DNMT3B), are essential for establishment of the DNA methylation patterns in early development (Jones, 2012). DNA methyltransferase 1 (DNMT1), together with DNMT3A and DNMT3B, is required for methylation maintenance (Jones & Liang, 2009).

Although, in general, imprinted genes are expressed from one parental allele and not the other, the silenced allele is not always completely silenced but may have residual activity (Horsthemke, 2010). Imprinting can also be tissue- and developmental stage –specific. The expression of the imprinted gene ubiquitin protein ligase E3A (*Ube3a*), for example, is suppressed to varying degrees in different tissues (Gustin et al., 2010). It has been shown that the imprinted expression that is present during early development may be either kept or lost in adulthood (Babak et al., 2015).

Models of imprinting

Insulator model

CTCF-binding sites can be found at imprinting clusters, where they act as insulators between gene promoters and enhancers (Bartolomei & Ferguson-Smith, 2011). One example of this type of regulation is seen at the *Igf2-H19* region. The ICR on the maternal allele is unmethylated and therefore allows binding of CTCF. The bound CTCF acts as an insulator, preventing enhancers from accessing the *Igf2* promoter. The enhancers can only access *H19* and therefore *H19* is expressed from the maternal allele. Conversely, the ICR on the paternal allele is methylated, preventing CTCF from binding and the insulator is not formed. As a result, *Igf2* is activated by enhancers while *H19* is repressed on the paternal chromosome (reviewed in Plasschaert & Bartolomei, 2014).

ncRNA model

Long non-coding RNAs (lncRNAs) are found in many imprinted gene clusters and have been found to regulate expression of nearby genes in the clusters (Koerner et al., 2009). One example is the *Kcnq1* (potassium voltage-gated channel, subfamily Q, member 1) locus, where the promoter of a lncRNA *Kcnq1ot1* (*Kcnq1* overlapping transcript 1) is located within the ICR of the imprinting cluster. The ICR on the maternal allele is methylated, which represses the lncRNA and the nearby genes are thus activated on the maternal chromosome. Conversely, the ICR on the paternal allele is unmethylated, allowing expression of the lncRNA, which in turn silences the nearby genes. It is not fully understood how the nearby genes are silenced, but it may involve repressive chromatin marks or preventing RNA polymerase II recruitment at promoters (Plasschaert & Bartolomei, 2014).

Loss of imprinting

Loss of imprinting (LOI) in humans can lead to imprinting disorders and cancer. LOI at *IGF2* occurs in a range of different tumor types, including colon, liver, lung, ovarian cancer and Wilms' tumor (Robertson, 2005). In 1994, Steenman and colleagues described how in Wilms' tumors with LOI, the maternal chromosome reverses to a paternal methylation pattern at the *H19-IGF2* region, causing increased levels of *IGF2*, decreased levels of *H19* and increased cell growth (Steenman et al., 1994). Imprinting control regions of paternally expressed 3 (*PEG3*), mesoderm specific transcript (*MEST*) and guanine nucleotide binding protein alpha stimulating complex locus (*GNAS*) are affected in breast, lung and ovarian cancers (Kim et al., 2015).

Imprinting disorders in humans often cause phenotypes with disturbed growth and neurological abnormalities (Walter & Paulsen, 2003). Heterogeneous and overlapping clinical features, heterogeneous molecular etiology including mosaicism, as well as multi-locus imprinting defects are also common features of imprinting disorders (Mackay & Temple, 2017). Imprinting disorders include Silver-Russell syndrome (SRS), Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), Prader-Willi syndrome (PWS), Temple syndrome (TS), Kagami-Ogata syndrome (KOS), transient neonatal diabetes mellitus (TNDM), pseudohypoparathyroidism type 1b (PHP1B) and Mulchandani-Bhoj-Conlin syndrome (MBCS) (Mackay & Temple, 2017). Opposite changes in an imprinted region can lead to imprinting disorder with contrasting phenotypes, such as growth restriction in SRS with hypomethylation of *H19-IGF2* intergenic DMR (*H19/IGF2*:IG-DMR) and overgrowth in BWS with hypermetylation of *H19/IGF2*:IG-DMR (Eggermann et al., 2016). Similarly, a paternal deletion of 15q11-q13 is seen in PWS, while a maternal deletion of the same region leads to AS (Buiting, 2010).

Genomic aberrations that cause imprinting disorders include UPD, chromosomal imbalances and point mutations. Imprinting disorders are also caused by epimutations, which are DNA methylation defects without genomic alterations.

These methylation changes can result from a defect in imprint erasure in primordial germ cells, defects in imprint establishment in sperm and oocytes, or defects in imprint maintenance in the embryo (Horsthemke, 2010).

Causes of human imprinting disorders

Uniparental disomy (UPD)

UPD is the abnormal inheritance of both copies of a chromosome from only one parent, as first described by Engel in 1980 (Engel, 1980). As imprinted genes are differentially expressed based on the parent-of-origin, receiving both copies of a chromosome from only one parent results in disrupted expression pattern of imprinted genes within the UPD region.

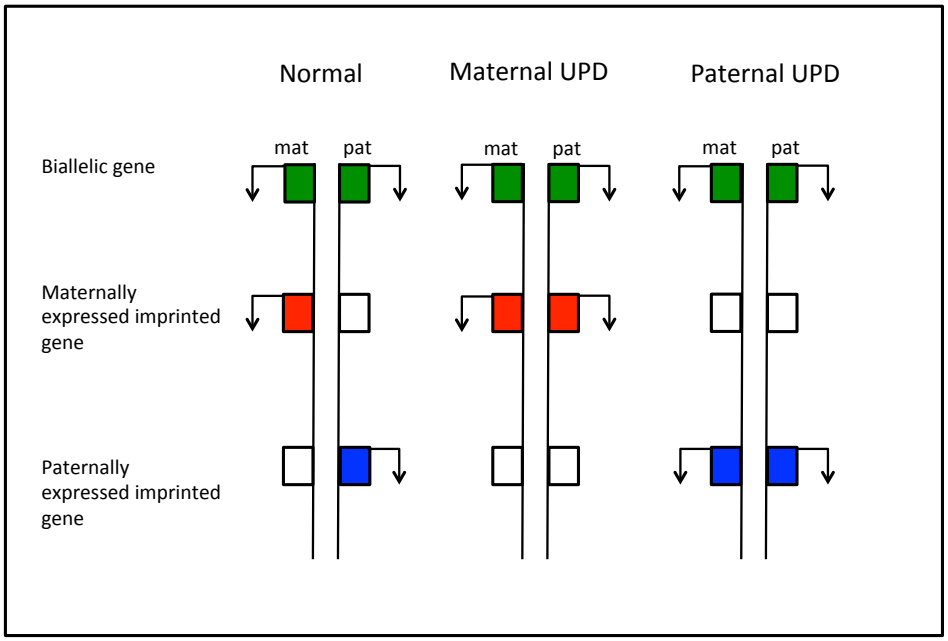


Figure 4. UPD affects the dosage of imprinted genes. Modified from Hitchins & Moore, 2002.

UPD can include a whole chromosome or just a part of a chromosome, in which case it is called segmental UPD (Kotzot, 2001). UPD can be either heterodisomic or isodisomic. In heterodisomy, both homologous chromosomes are transmitted from the same parent, while isodisomy is the result of two copies of one parental chromosome (Mergenthaler et al., 2000). Uniparental isodisomy can also cause recessive allele penetrance, so that a recessive disease will manifest in a person whose only one parent is a carrier of the disease allele (Spence et al., 1988).

Different types of events can lead to UPD. UPD of a whole chromosome can be the result of a trisomy or monosomy rescue, gamete complementation or post-fertilization error (Kotzot, 2008a). When one parental gamete is disomic and the other monosomic, this situation will lead to trisomy. However, if the extra chromosome from a trisomic zygote is expelled, this is called trisomy rescue and it will result in either a normal biparental zygote or lead to UPD if the two chromosomes left in the zygote came from the same parent. In monosomy rescue, monosomic and nullisomic gametes will combine, creating a monosomic zygote. As a result of mitotic duplication, UPD will form. Gamete complementation, the situation where disomic and nullisomic gametes combine, will also lead to UPD. In postfertilization errors two normal monosomic gametes combine, resulting in a normal biparental zygote, but a mitotic segregation error will lead to trisomy or monosomy, after which either a loss or duplication of a chromosome will lead to UPD (Figure 5).

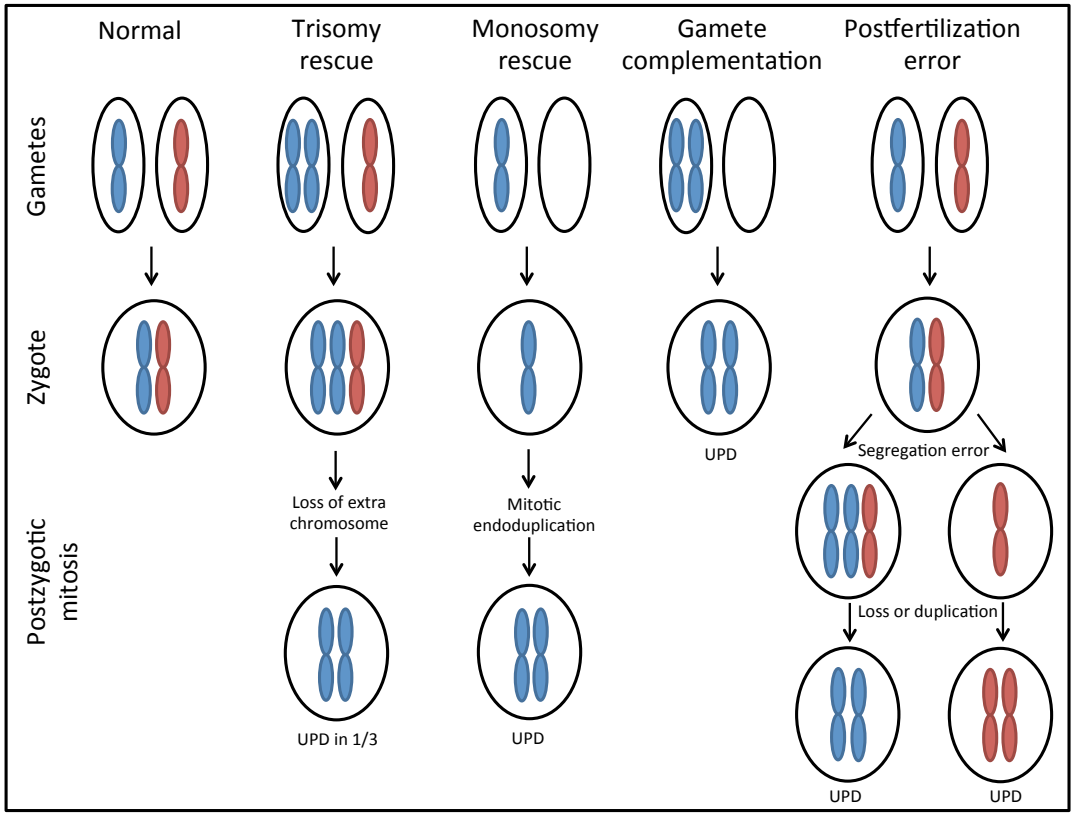


Figure 5. Causes of UPD. Modified from Tollefsbol, 2011.

Deletions and duplications

Chromosomal deletions and duplications are common in some imprinting disorders and rare in others. In AS and PWS, chromosomal imbalances affect the majority of the patients. 70% of AS patients have a deletion on the maternal chromosome 15 (15q11.2), while a paternal deletion of the same region of chromosome 15 is responsible for 70% of PWS cases. In TNDM, 40% of the patients have a paternal duplication on chromosome 6 (6q24). Maternal and paternal deletions of 14q32 affect 15 and 10% of KOS and TS, respectively. Chromosomal aberrations are rare in SRS, representing less than 1% of the patients (reviewed in Mackay & Temple, 2017).

Point mutations

Genomic point mutations are responsible for some cases of imprinting disorders. These mutations may affect regulation of ICRs or genes involved in establishment or maintenance of DNA methylation (Tollefsbol, 2011). In BWS, cyclin dependent kinase inhibitor 1C (*CDKN1C*) mutations occur in 8% of the patients, including familial and sporadic cases (Brioude, 2015). Approximately 5% of TNDM patients have recessive mutations of zinc-finger protein 57, *ZFP57,* that result in loss of methylation at the TNDM locus and other imprinted loci (Boonen et al., 2013). The mouse *Zfp57* has a role in both methylation establishment and maintenance during development (Boonen et al., 2013).

Epimutations - abnormal DNA methylation without genomic alterations

In some imprinting disorders such as SRS and BWS, the majority of cases are caused by altered methylation patterns at ICRs. The 11p15 region, implicated in both SRS and BWS, can be divided into two domains. The telomeric domain contains the paternally expressed *IGF2* and maternally expressed *H19*, and its imprinting is controlled by *H19/IGF2*:IG-DMR (also known as *ICR1, IC1, H19* DMR). Loss of methylation at the *H19/IGF2*:IG-DMR is the most common molecular cause of SRS and results in reduced *IGF2* expression and increased *H19* expression (Wakeling et al., 2017). The opposite change in the *H19/IGF2* IG-DMR, gain of methylation, is the molecular cause of BWS in 5-10% of the patients (Eggermann et al., 2014a). The centromeric domain contains *CDKN1C* and a lncRNA *KCNQ1OT1*, controlled by the *KCNQ1OT* transcription start site DMR (*KCNQ1OT1*:TSS-DMR, also known as *ICR2*). Loss of methylation at the *KCNQ1OT1*:TSS-DMR accounts for BWS in 50-60% of patients (Eggermann et al., 2014a).

Silver-Russell Syndrome (SRS)

Silver-Russell syndrome is an imprinting disorder characterized by pre- and postnatal growth restriction and variable clinical features. SRS was first discovered independently by Silver and colleagues (Silver et al., 1953), and Russell (Russell, 1954), who described children with growth restriction and additional clinical characteristics, such as body asymmetry and specific facial features. In addition to poor prenatal and postnatal growth, the main additional features in SRS include relative macrocephaly, protruding forehead, body asymmetry and feeding difficulties (Wakeling et al., 2017). SRS is very heterogeneous in both phenotype and molecular etiology. The incidence of SRS is estimated to be 1/3000 to 1/100 000 (Abu-Amero et al., 2008). In a study of 386 SRS patients, the mean adult height was reported 151 +/- 7.8 cm for males and 139.9 +/- 9.0 cm for females (Wollmann et al., 1995).

Diagnosis

The diagnosis of SRS can be challenging due to the heterogeneity of the phenotype. Multiple clinical scoring systems have been developed and used in diagnosis. The first international SRS consensus statement (Wakeling et al., 2017), presents the Netchine-Harbison clinical scoring system (NH-CSS)(Azzi et al., 2015) for investigation and diagnosis of SRS. It is more sensitive (98%) than some of the other scoring systems (Wakeling et al., 2017). NH-CSS uses six clearly defined criteria (Table 1). Clinical SRS diagnosis is given when at least four of the criteria are fulfilled. However, in the original publication of the scoring system, the NH-CSS system missed one SRS patient with UPD(7)mat, who only fulfilled three of the criteria (Azzi et al., 2015). Molecular testing for SRS is therefore recommended when only three of the criteria are met (Wakeling et al., 2017).

SRS clinical criteria	Definition
Prenatal growth retardation	Birth length and/or weight ≤-2 SDS for gestational age
Postnatal growth retardation	Height at 24 ±1 months ≤−2 SDS or height ≤−2 SDS from midparental target height
Relative macrocephaly at birth	Head circumference at birth at least 1.5 SDS above birth weight and/or length SDS
Protruding forehead	Forehead that projects beyond the facial plane on a side view as a toddler
Body asymmetry	Leg length discrepancy of at least 0.5 cm or arm asymmetry or leg length discrepancy less than 0.5 cm with at least two asymmetrical body parts (one non-face)
Feeding difficulties and/or low BMI	Use of a feeding tube or cyproheptadine for appetite stimulation and/or BMI ≤-2 SDS at 24 months

Table 1. Clinical criteria for diagnosis of SRS. According to Wakeling et al., 2017 and Azzi et al., 2015.

Additional clinical features of SRS

In addition to the main features listed in the diagnostic criteria, SRS patients have many features that are present at varying frequencies. Triangular face is seen in 94% of SRS patients, fifth finger clinodactyly in 75%, micrognathia in 62%, low muscle mass in 56%, excessive sweating in 54%, low-set or posteriorly rotated ears in 49%, down-turned mouth in 48%, high-pitched or squeaky voice in 45%, speech delay in 40%, male genital abnormalities in 40%, motor delay in 37%, syndactyly of toes in 30%, among other features (Wakeling et al., 2017). These features are not specific to SRS, but are present at a higher frequency SRS than in non-SRS patients. Global developmental delay or learning difficulties have been described in some SRS patients, especially in the UPD(7)mat subgroup. (Wakeling et al., 2017)

Clinical management

SRS is an indication for growth hormone (GH) treatment, which increases the adult height of the patients. The gain in height has been reported similar in SRS children and non-SRS children who are small for gestational age (SGA), with a total height gain of 1.3 SDS in SRS and 1.26 SDS in non-SRS SGA children (Smeets et al., 2016), however noting that final adult height of SRS patients will be lower than that of the non-SRS SGA patients because of more severe growth restriction and earlier onset of puberty. A trend has been reported towards UPD(7)mat patients having a greater benefit from GH treatment than those with 11p15 epimutation (Binder et al., 2008)(Smeets et al., 2016).

SRS children often suffer from malnutrition, which may limit the benefits of GH treatment on height (Marsaud et al., 2015). Nutritional management is therefore essential and may include nutritional support, such as enriched diet, oral nutritional supplements and enteral feeding (Marsaud et al., 2015). However, because rapid weight gain in children born SGA is associated with an increased metabolic and cardiovascular risk later in life, it is essential to avoid overfeeding SRS children (Wakeling et al., 2017).

Other clinical management strategies in SRS children include prevention of hypoglycemia, monitoring for signs of premature adrenarche and insulin resistance, assessment of neurocognitive problems including speech delay, and management of orthopedic problems, such as asymmetry and limb anomalies. (Wakeling et al., 2017). A recent study also suggests that SRS patients experience psychosocial issues, including appearance-related concerns, and could benefit from early psychosocial intervention (Ballard et al., 2018).

Differential diagnosis

As features of SRS are heterogeneous and overlap with other growth disorders, differential diagnosis can be challenging. Syndromes that are very close in clinical phenotype are Mulibrey nanism (MUscle, LIver, BRain, EYes nanism) and 3-M (Miller, McKusick, and Malvaux) syndrome. Mulibrey nanism and SRS patients both have intrauterine growth failure, lack of postnatal catch-up growth,

feeding difficulties and similar facial dysmorphisms. Features that may help distinguish SRS and Mulibrey from each other are clinodactyly, micrognathia, down-turned mouth corners and asymmetry, which are characteristic of SRS, and hepatomegaly, heart failure (due to pericardial constriction or cardiomyopathy), yellow dots in ocular fundi and fibrous dysplasia of long bone, which do not occur in SRS patients (Karlberg et al., 2004). 3-M syndrome and SRS patients both have pre- and postnatal growth restriction, relatively large head and triangular face, and in multiple cases patients initially diagnosed with autosomal recessive SRS have been later found to have mutations related to 3-M syndrome (Akawi et al., 2011). Other syndromes that have been suggested for differential diagnosis in patients with normo- or macrocephaly are SHORT (short stature, hyperextensibility of joints, ocular depression, Rieger anomaly and teething delay) syndrome, Floating harbour syndrome and IMAGe (intrauterine growth restriction, metaphyseal dysplasia, congenital adrenal hypoplasia, genital anomalies) syndrome, and for patients with relative microcephaly, Bloom syndrome, Nijmegen breakage syndrome, Microcephalic osteodysplastic primordial dwarfism type II, Meier-Gorlin syndrome, *IGF1R* mutation or deletion and insulin-like growth factor (*IGF1*) mutation (Wakeling et al., 2017).

Etiology of SRS

Maternal UPD of chromosome 7 [UPD(7)mat]

Maternal uniparental disomy for chromosome 7, UPD(7)mat, was the first molecular diagnosis suggested in SRS by Kotzot and colleagues in 1995, when they found three cases of UPD(7)mat among 35 patients with SRS or primordial growth restriction (Kotzot et al., 1995). Previously, UPD(7)mat had been detected in two patients with short stature and cystic fibrosis (CF), where only the mother was a carrier of CF (Spence et al., 1988)(Voss et al., 1989) and in one patient with short stature and a homozygous mutation for collagen type I alpha 2 chain (*COL1A2*), where only the mother was a carrier for the mutation (Spotila et al., 1992). UPD(7)mat can therefore lead to short stature, as well as recessive disease allele penetrance. Subsequent studies include findings of UPD(7)mat in

6% (Preece et al., 1997), 10% (Eggermann et al., 1997) and 12.5% of SRS patients (Hannula et al., 2001b).

There are multiple imprinted genes on chromosome 7, and it is thought that the disrupted imprinting pattern resulting from two maternal copies of chromosome 7 causes the SRS phenotype. Although UPD in SRS has been researched widely, it is still unclear which imprinted genes in chromosome 7 cause SRS. Segmental UPDs and copy number variants (CNVs) that have been reported in SRS have suggested candidate gene regions, such as growth factor receptor bound protein 10 (*GRB10,* at 7p12.1) and *MEST* (at 7q32). Reported segmental UPD(7)mat cases include UPD(7q31-qter)mat (Hannula et al. 2001a), mosaic UPD(7q21-qter)mat in a patient with severe intrauterine and postnatal growth restriction (Reboul et al., 2006), and two patients with UPD(7q11.2-qter)mat with slight SRS features (Eggermann et al., 2008). Gerbrands and colleagues recently also reported of a SRS patient with cystic fibrosis and segmental maternal isodisomy of chromosome 7, covering at least 7q31-34 (Gerbrands et al., 2017).

The clinical phenotype of UPD(7)mat is milder than that of other SRS patients (Hannula et al., 2001b). Binder and colleagues have reported that birth length was significantly higher in SRS patients with UPD(7)mat than those with 11p15 LOM (Binder et al., 2008). Bruce and colleagues have reported lower incidence of certain features such as asymmetry, clinodactyly and down-turned mouth corners in UPD(7)mat patients and a greater incidence of speech delay, feeding difficulties and a high-pitched voice, among other findings (Bruce et al., 2009). Wakeling and colleagues also reported less asymmetry, clinodactyly and congenital anomalies in UPD(7)mat patients than in 11p15 LOM patients, while learning difficulties and referral for speech therapy was more common (Wakeling et al., 2010). UPD(7)mat patients have been reported with significantly higher maternal and paternal ages (Bruce et al., 2009), which increase the risk for uniparental disomy (Robinson et al., 1993).

Interestingly, while UPD(7)mat results in a clear growth restriction phenotype, in UPD(7)pat cases no growth abnormalities have been reported (Höglund et al.,

1994)(Pan et al., 1998)(Le Caignec et al., 2007).

Loss of methylation at 11p15 (11p15 LOM)

Gicquel and colleagues were the first to report loss of methylation at the *H19/IGF2*:IG-DMR in five out of nine SRS patients (Gicquel et al., 2005), but the same region had been previously found to be associated with maternal duplications in patients with growth restriction (Fisher et al., 2002) and SRS (Eggermann et al., 2005). Later studies found 11p15 LOM in approximately 20-60% of SRS patients (Eggermann et al., 2006)(Bliek et al., 2006)(Schönherr et al., 2006).

11p15 LOM is associated with a more severe or classic SRS phenotype than other SRS patients. Asymmetry is more common in 11p15 LOM than in other SRS patients (Netchine et al., 2007)(Binder et al., 2008)(Bruce et al., 2009)(Bartholdi et al., 2009)(Wakeling et al., 2010). Birth weight, birth length and postnatal body mass index (BMI) were significantly lower, and a prominent forehead and relative macrocephaly were significantly more frequent in SRS patients with 11p15 LOM than in patients without 11p15 LOM (Netchine et al., 2007). Also Binder and colleagues reported typical facial features more frequent in 11p15 LOM SRS than other SRS patients (Binder et al., 2008). Fifth finger clino- and brachydactyly, cryptorchidism, syndactyly of toes, and down-turned mouth corners were more common in 11p15 LOM patients compared to UPD(7)mat (Bruce et al., 2009). Also Wakeling and colleagues reported fifth finger clinodactyly and congenital anomalies as more common in patients with 11p15 LOM than in patients with UPD(7)mat (Wakeling et al., 2010). Patients with extreme 11p15 LOM were reported to have abnormally high lumbar vertebrae, lumbar hypomobility, elbow subluxations and specific hand and foot anomalies (Bruce et al., 2009).

Gicquel and colleagues found low expression of *IGF2* in skin fibroblasts of 11p15 LOM patients (Gicquel et al., 2005). Serum *IGF2* levels, however, have been shown to be normal in SRS patients (Binder et al., 2006)(Netchine et al., 2007),

and it was proposed that the lack of changes in *IGF2* level in blood is due to *IGF2* being regulated in a developmental- and tissue-specific way (Netchine et al., 2007). In 2014, Azzi and colleagues reported a significant correlation between the methylation index of *IGF2* and fibroblast *IGF2* expression, supporting that *IGF2* expression is regulated by methylation of the *H19/IGF2*:IG-DMR. They also showed that methylation levels of *H19/IGF2*:IG-DMR may differ between cells from different tissues, suggesting multiple mosaic, tissue-specific epigenotypes within individual patients (Azzi et al., 2014).

It has also been reported that 3.8% (3 out of 77)(Begemann et al., 2011), 4% (3 out of 74)(Azzi et al., 2009) and 3 out of 6 patients (Poole et al., 2013) of SRS patients with confirmed 11p15 ICR1 hypomethylation also have ICR2 hypomethylation, which is usually associated with BWS.

Multi-locus imprinting distubance (MLID)

MLID, also known as multilocus loss of methylation (multilocus LOM), multilocus methylation defects (MLMD), multilocus hypomethylation syndrome (MHS), multilocus hypomethylation (MLH) or hypomethylation of multiple imprinted loci (HIL), was described in SRS patients in 2009 by Azzi and colleagues (Azzi et al., 2009), where 7 out of 74 SRS patients were discovered to have loss of methylation at regions other than 11p15. Turner and colleagues (Turner et al., 2010) also found hypomethylation of multiple imprinted loci in 2 out of 23 patients with 11p15 LOM. Later studies have found MLID in 17% (Poole et al., 2013), 7.1% (Eggermann et al., 2014b) and 11.4% (Azzi et al., 2015) of SRS patients with 11p15 LOM. A genome-wide study has detected 73% of SRS patients with additional aberrant methylation marks outside of 11p15 LOM (Kannenberg et al., 2012). Azzi and colleagues (Azzi et al., 2009) did not find significant differences in phenotype between 11p15 SRS patients with or without MLID. Similar results were obtained by Poole and colleagues (Poole et al., 2013), when they compared the clinical phenotypes of 11p15 LOM SRS patients with and without MLID, and found no statistically significant difference between the two groups, although developmental delay and atypical SRS features were more

common in patients with MLID. The effect of MLID on the clinical phenotype of SRS patients remains to be clarified. MLID is generally mosaic and imprinting patterns vary between tissues, which may indicate that MLID arises post-fertilization (Mackay & Temple, 2017). MLID has been shown to affect both paternally and maternally imprinted genes, with loss and gain of methylation, and in a mosaic pattern, suggesting origin in maintenance rather than establishment of imprinting patterns (Eggermann, 2014b).

Copy number variations (CNVs) and other rare findings

Many different CNVs have been reported in SRS, including various maternal duplications of 11p15 (reviewed in Begemann et al., 2012 and Fokstuen & Kotzot, 2014), duplication of 7p12.1-p13 (Joyce et al., 1999), maternal duplication of 7p11.2-p13 (Monk et al., 2000), and many others at locations outside chromosome 7 or 11 (Fokstuen & Kotzot, 2014). In a review of all CNVs reported in SRS patients up to 2013, Fokstuen and Kotzot evaluated clinical characteristics of the reported patients and concluded that most of the chromosomal arrangements (except for duplication of 11p15) do not justify SRS diagnosis in the patients (Fokstuen & Kotzot, 2014).

In single cases of SRS or SRS-like patients, mosaic UPD(11)mat (Bullman et al., 2008), *CDKN1C* gain-of-function mutation in a family with maternally transmitted SRS (Brioude et al., 2013) and paternally transmitted *IGF2* loss-of-function mutation (Begemann et al., 2015) have been reported.

Clinical SRS with unknown etiology

A molecular cause can be identified in approximately 60% of patients with SRS (Wakeling et al., 2017). For those patients who fulfill the SRS criteria but have negative results in molecular testing, the term clinical SRS is used. Alternative terms for clinical SRS in literature include idiopathic SRS (Spengler et al., 2010) and SRS of unexplained etiology (Kotzot, 2008b). Identification of additional molecular causes in clinically diagnosed SRS is important (Wakeling et al., 2017).

Imprinted genes in normal growth

While the effect of imprinting defects on growth is clearly demonstrated in imprinting disorders such as SRS, more subtle changes in imprinted genes have been found to be associated with various growth parameters in the normal population. Single nucleotide polymorphisms (SNPs) in the *IGF2R, IGF2* and *H19* gene regions have been found to be associated with birth weight (Adkins et al., 2010). A maternally inherited repeat sequence (RS1) of the imprinted *PHLDA2* (pleckstrin homology like domain family A member 2) causes an increase of birth weight and head circumference (Ishida et al., 2012). The expression levels of *IGF2*, *IGF2R* and *H19* in chorionic villus samples at approximately 12 weeks gestation are correlated with crown-rump length (Moore et al., 2015), demonstrating growth regulation by imprinted genes early in development. A SNP in the *IGF2-H19* region is associated with birth length and adult height in a parent-of-origin dependent manner (Benonisdottir et al., 2016), showing the effect of imprinted genes on height during development as well as on the final adult height in the normal population.

The study of the effect of imprinted genes on growth is complicated by the dual role of imprinted gene expression as restrictive or promoting of growth. For example, the observation of overexpression of the imprinted paternally expressed 10 (*PEG10*) in IUGR (intrauterine growth restriction) placentas is thought to result from a compensatory mechanism trying to protect a fetus from growth restriction by promoting growth (Piedrahita, 2011).

Environmental effects on imprinted genes and later disease development

Low birth weight is associated with many adult-onset diseases, such as hypertension (Huxley et al., 2000) and coronary artery disease (Barker et al., 1989). Adverse environmental conditions during critical times of development can predispose an individual to later development of disease. For example, exposure to famine in fetal period and infancy is associated with a significantly increased risk of hypertension and impaired glucose tolerance in adulthood

(Hult et al., 2010). Preterm birth has also been linked with increased blood pressure (de Jong et al., 2012), insulin resistance (Hofman et al., 2004) and elevated plasma cortisol levels (Szathmari et al., 2000).

The effects of environmental exposures may be mediated through epigenetic mechanisms. These environmental factors occurring early in development can cause changes in DNA methylation that persist throughout life (Heijmans et al., 2008). In rats, maternal care during postnatal period affects stress responsivity of the offspring through methylation changes in glucocorticoid receptor (GR) promoter (Weaver et al., 2004).

Imprinted genes, in particular, may be sensitive to environmental effects (Waterland & Jirtle, 2004). Increased methylation levels of the imprinted *IGF2* have been reported in infants born to smokers compared to infants born to nonsmokers (Murphy et al., 2012). DNA methylation levels of *IGF2* are also reported lower in people who were prenatally exposed to famine during the Dutch Hunger Winter in 1944-45 (Heijmans et al., 2008). Also postnatal exposures, such as early lead exposure in childhood, results in DNA methylation changes in DMRs of the imprinted *PEG3, IGF2/H19* and *PLAGL1/HYMAI* (PLAG1 like zinc finger 1/hydatidiform mole associated and imprinted) measured in adulthood (Li et al., 2016). Assisted reproductive technologies have also been associated with increased incidence of imprinting disorders (Allen & Reardon, 2005), but the evidence is not conclusive and the association could be at least in part be explained by the reproductive disease of the parents (Pinborg et al., 2016)

AIMS OF THE STUDY

This thesis aimed to explore the genetic and epigenetic factors contributing to SRS and human growth, as well as epigenetic changes in preterm-born very low birth weight individuals.

The specific aims were

I to search for genomic aberrations in SRS and SRS-like patients of unknown etiology

II to determine DNA methylation changes in the *IGF2* gene 20 years after preterm birth at very low birth weight in individuals with elevated cardiovascular risk factors

III to find new imprinted genes by using DNA methylation differences in UPD(7)mat patients compared to controls and UPD(7)pat

IV to find new, shared DNA methylation changes among different subtypes of SRS and to find out whether those changes are associated with height in the general population

MATERIALS AND METHODS

Patients

Study I: 22 patients with SRS were included in the study. 10 of the SRS patients had 11p15 LOM and 12 had clinical SRS. Also parents of the children were included in the study. The patients were recruited mainly from the Hospital for Children and Adolescents, Helsinki University Central Hospital, Finland.

Study II: 158 young adults born preterm at very low birth weight (VLBW) were from the Helsinki Study of Very Low Birth Weight Adults that includes patients who were born preterm at very low birth weight and were treated at the Neonatal intensive care unit of the Children's Hospital of the Helsinki University Central Hospital. The 161 control subjects were matched for sex, age and birth hospital and were born at term with birth weight more than -2SD. The age range for the individuals studied at the time when DNA methylation was measured was from 18 to 27 years.

Study III: 9 UPD(7)mat patients, including one patient with segmental UPD(7q31-qter), ten controls and one individual with UPD(7)pat were studied. Ten unrelated individuals of normal height were used as controls. The SRS patients were recruited from the Hospital for Children and Adolescents, Helsinki University Central Hospital, Finland. Additionally three patients were referred from the Oulu University Central Hospital and one from the Päijät-Häme Central Hospital, Finland. Samples for parent-of-origin allele-specific expression analysis were obtained from nine parent-child trios from the growth restriction study cohort, in which the child of the trio had previously been confirmed not to have UPD.

Study IV: 44 SRS patients, of whom 21 patients had 11p15 LOM, 10 patients had UPD(7)mat and 13 patients had clinical SRS (i.e. no findings of 11p15 LOM or UPD(7)mat) were studied. One of the 10 UPD(7)mat patients had segmental UPD(7q31-qter)mat. One UPD(7)pat sample was also included. The control

samples were obtained from ten adults of normal height and six additional samples from adults that were healthy blood donors. 39 patients with severe growth restriction of unknown etiology (SGR) were included in a targeted analysis of *HOXA4* hypomethylation. Majority of the SGR patients were born SGA and all of them had postnatal growth restriction. The SRS and SGR patients were recruited mainly from the Hospital for Children and Adolescents, Helsinki University Central Hospital, Finland. Methylation data from 227 healthy children of the BAMSE (Swedish abbreviation for Children, Allergy, Milieu, Stockholm, Epidemiology) cohort, a birth cohort of children born in Stockholm between 1994 and 1996), was used to perform correlation analysis of height and *HOXA4* methylation level.

The studies were conducted in accordance with the principles of the declaration of Helsinki. Informed consent was obtained from all participants. The studies were approved by the appropriate ethical review boards: the Ethical Review Board of the Hospital for Children and Adolescents, Helsinki University Central Hospital, Helsinki, Finland, the Ethics Committee of the Helsinki and Uusimaa Hospital District, Finland and the Ethical Review Board North at Karolinska Institutet, Sweden.

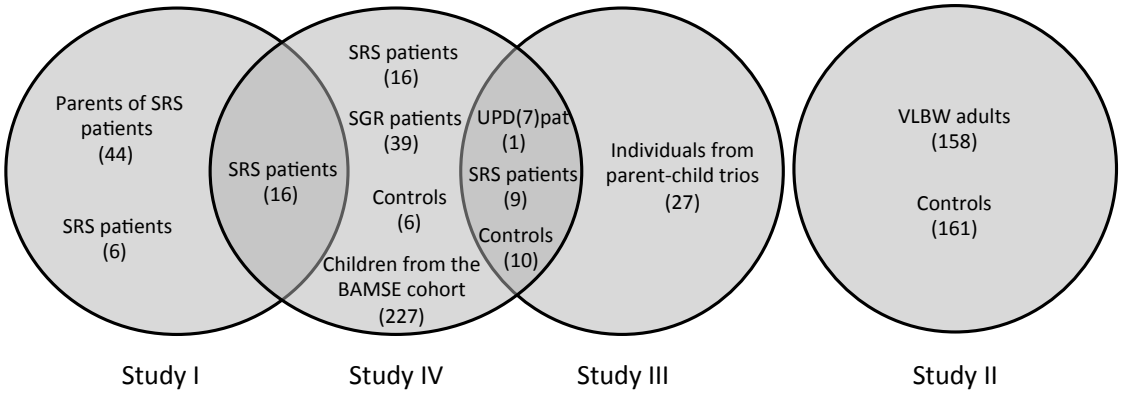


Figure 6. Patients and controls from all four studies.

Sample processing

DNA extraction (Studies I, II, III and IV)

DNA from the participants' whole blood samples was extracted using FlexiGene DNA Kit (Qiagen) or QIAamp DNA Blood Maxi Kit (Qiagen).

Bisulfite conversion (Studies II, III and IV)

Bisulfite conversion of DNA was performed in order to prepare the samples for DNA methylation analysis with EpiTYPER, pyrosequencing and Illumina 450K HumanMethylation Bead chip in studies II, III and IV. Bisulfite treatment converts unmethylated cytosine to uracil, but leaves 5-methylcytosine unaffected, allowing distinction between the unmethylated and methylated cytosines. 500 ng of DNA was converted with the EZ-96 Methylation Kit (Zymo research corporation) for studies II, III and IV. The EpiTect Bisulfite Kit (Qiagen) was used for bisulfite conversion of 2 ug of DNA for the pyrosequencing study in Study III.

RNA extraction (Study III)

Whole blood was collected into PAXgene Blood RNA tubes (PreAnalytix, GmbH) and RNA was extracted using the PAXgene Blood miRNA Kit (PreAnalytix, GmbH). Bioanalyzer (Agilent Technologies) was used to check the quality of the RNA samples. RIN (RNA integrity number) indicates the level of quality of the RNA sample. All of the samples had RIN above 8 and were therefore of sufficient quality to be included in the analyses.

Methods for studying genetics

Affymetrix genotyping (Study I)

In addition to genotyping, Affymetrix genotyping arrays can be used for both DNA copy number analysis and detection of UPDs. The GeneChip Mapping 250K Sty array enables study of 238 000 SNPs distributed across the genome. The array contains allele-specific oligonucleotide hybridization probes, located at specific positions on the array that are complementary to the sequence of the studied SNP region. The protocol includes digestion of genomic DNA with a restriction enzyme Sty I (a restriction enzyme purified from E. coli), ligation of adaptors to the ends of the restriction fragments, PCR (polymerase chain reaction) amplification of the fragments, fragmentation of the PCR products with DNAse I (deoxyribonuclease I), biotinylation of the fragments and hybridization of the fragments to the arrays. When the arrays are scanned, genotypes are determined based on hybridization signal intensities from allele-specific probes (Matsuzaki et al., 2004). Copy number changes can be evaluated by comparing the signal intensity values against a reference set of data (Winchester et al., 2009). The array was used in Study I to detect copy number changes in SRS and SRS-like patients.

Polymerase chain reaction (PCR)

PCR is a technique by which a small amount of a specific target sequence of DNA can be amplified. It requires two oligonucleotide fragments (primers) that are complementary to the target DNA sequence. Each cycle of different temperatures causes separation of the DNA strands, annealing of primers to the complementary sequence and an extension by DNA polymerase, resulting in doubling of the amount of DNA. As the cycles are repeated, the target sequence accumulates exponentially, approximately 2^n, n being the number of cycles (Saiki et al., 1988). PCR was used in all four studies.

Methods for studying epigenetics

Pyrosequencing (Study III)

Pyrosequencing technology can be used for studying DNA methylation, among other uses. A several hundred base pair segment is PCR amplified, with one primer biotinylated. The biotinylated PCR products are immobilized onto streptavidin-coated sepharose beads. After separation of the strands, the biotinylated single strand hybridizes with a sequencing primer and DNA polymerase catalyzes the addition of dNTPs (deoxyribonucleotide triphosphates). Each addition is accompanied by a release of pyrophosphate, which through several reactions causes emission of visible light that is detectable in a charge coupled device camera. Nucleotide sequence can be detected from the signal peaks (reviewed in Schock & Traeger, 2011). In Study III, pyrosequencing was used to validate the methylation status of specific regions by first converting the samples by bisulfite treatment. The technology provides a methylation percentage for each individual CpG site.

EpiTYPER (Studies II and IV)

EpiTYPER technology enables the study of DNA methylation of CpG sites within designed amplicons of regions of interest. EpiTYPER requires bisulfite treatment of the DNA samples. Unmethylated cytosines are converted into uracils and methylated cytosines remain unchanged. PCR is run, after which the PCR product is transcribed into an RNA transcript and cleaved base specifically by an endonuclease. The cleavage products are analyzed by matrix assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS). The mass spectrometer differentiates between methylated and unmethylated CpGs by detecting the mass difference of 16 Da (Daltons) per CpG site (G vs. A). Relative methylation level can be calculated by comparing the intensities of the signals coming from the methylated and unmethylated cleavage products. Each analyzed CpG unit may contain one or more CpG sites. When there are multiple CpGs in one CpG unit, the mass difference detected by the mass spectrometer

will be in multiples of 16 Da (Ehrich et al., 2005). In Studies II and IV, EpiTYPER was used for targeted methylation studies of regions of interest.

Genome-wide methylation analysis with Illumina 450K BeadChip assay (Studies III and IV)

The Illumina Human Methylation BeadChip is a technology that enables the study of DNA methylation of more than 450 000 individual CpG sites across the genome, covering 99% of RefSeq genes and 96% of CpG islands. The BeadChip requires use of bisulfite-converted DNA. Two assay types, Infinium I and Infinium II, are included in the microarray. Infinium I assay consists of two bead types, one for methylated CpG and one for unmethylated CpG. In Infinium II assay, one bead type corresponds to each CpG locus and the methylation state is detected by single base extension. In the assay protocol, bisulfite treated samples are whole genome amplified, fragmented and hybridized to the bead chips. After extension and staining steps, the BeadChips are imaged. Methylation level is calculated based on the intensities of the methylated and the unmethylated signal (Bibikova et al., 2011). In studies III and IV, the Illumina 450K BeadChip was used to study genome-wide methylation changes in UPD patients and SRS subgroups.

Methods for studying gene expression and copy number changes

Quantitative real-time PCR (Study I and III)

Quantitative real-time PCR (qPCR) is a technique based on real-time monitoring of each cycle of PCR. qPCR can be used, for example, to study the expression level of genes or to detect CNVs in genomic DNA samples. In general, qPCR can be performed using either the fluorescent dye SYBR-Green, that binds to double-stranded DNA (Ponchel et al., 2003), or TaqMan assays, that use a hybridization probe labeled with two different fluorescent dyes (Heid et al., 1996).

SYBR-Green based qPCR was used in Study I to verify CNVs that had been detected with the Affymetrix 250K genotyping array. PCR amplicons located within the deleted or duplicated regions were used to verify the copy number change by comparing the C_T values of the patient DNA samples to the C_T values of the control DNA samples, a method originally described by Weksberg and colleagues (Weksberg et al., 2005).

Taqman gene expression assays were used in Study III to quantify expression levels of genes that had been identified as potential imprinted genes, and were therefore predicted to show significant gene expression differences between UPD(7)mat samples, UPD(7)pat sample and controls.

Results of the qPCR experiments were calculated with the comparative C_T method (Schmittgen and Livak, 2008).

Parent-of-origin allele-specific expression analysis (Study III)

Parent-child trios can be used to verify that an allele is being expressed from only one parental chromosome. The requirement is to obtain DNA samples from a child and his/her two parents, and an RNA sample from the child. Individuals with UPDs of the studied region must be excluded from the analysis. Monoallelic expression can be tested by using SNPs that are located in an exon of the studied gene. SNPs that are heterozygous in the child and homozygous in at least one parent can be used in the analysis. The child's RNA is converted into cDNA (complementary DNA) and sequenced. If the child's cDNA confirms monoallelic expression, maternal or paternal specific expression of the gene may be deduced by looking at the parental genotypes. The described strategy was used in Study III to verify parent-of-origin allele-specific expression of potential imprinted genes.

Data analysis, statistical methods and data visualization

Data analysis for the Illumina 450K BeadChip (Study III and IV)

In Study III, the raw data was analyzed using the BioConductor package lumi in R v. 2.13 (Du et al., 2008). Adjustments for color channel imbalance and background noise were made, and the data was normalized using the quantile method. Differential methylation for each probe was calculated by a linear model and by pair-wise comparisons by empirical Bayes t-test on the normalized M-values (Smyth, 2005). The M-values are useful for assessing especially high and low methylation levels.

In Study IV, the raw data was analyzed using the minfi package (Aryee et al., 2014). The data was normalized using the subset-quantile within array normalization (SWAN) method (Maksimovic et al., 2012). All probes that overlapped with known SNPs and those with reported cross-hybridization problems were removed from the analysis. The ComBat method was used to remove batch effects (Johnson et al., 2007). CpGs with differential methylation were obtained by using linear models and pair-wise comparisons by empirical Bayes t-test using the limma package (Smyth, 2005). Further analysis proceeded with CpG sites that had a p-value < 0.05 after Benjamini and Hochberg correction for multiple testing.

Filtering the genome-wide methylation data (Study III and IV)

In order to best utilize the genome-wide methylation data, it was necessary to design a filtering approach by which it is possible to obtain information on specific changes in the methylation landscape.

In Study III, a filtering system was created to detect differentially methylated regions on chromosome 7. The filtering system was based on three criteria that were assumed to be true for differentially methylated regions: 1) the median methylation levels for UPD(7)mat, controls and UPD(7)pat for a CpG were either in ascending or descending order, 2) differential methylation between

UPD(7)mat and controls was statistically significant (p<0,05) and 3) there were at least three adjacent probes that passed criteria 1 and 2.

In Study IV, a filtering system was created in order to search for common differentially methylated regions among all three subgroups of SRS. This filtering system fulfilled two criteria: the methylation level for each SRS subgroup was statistically significantly different from the controls for a given CpG and there were at least three such CpG sites adjacent to each other.

In both studies, the filtering system was designed to detect stretches of differentially methylated CpGs, similar to the DMRs of the known imprinted genes, rather than single differentially methylated CpGs.

Data analysis for Affymetrix array (Study II)

Affymetrix GeneChip Genotyping Analysis Software (GTYPE 4.1) was used for determining genotypes, average heterozygosity estimates and pedigree checks. SNPTrio, an analysis tool for SNP data from trios, was used for identifying Mendelian inconsistencies (Ting et al., 2007). Copy Number Analyzer for Affymetrix GeneChip Mapping 2.0 (CNAG 2.0) was used for identifying copy number variation and loss-of-heterozygosity regions (Nannya et al., 2005). Also the Aroma.Affymetrix package in R was used to identify copy number variations (Bengtsson et al., 2008).

Methylation status of individuals (Study IV)

Normal methylation level for each *HOXA4* CpG of the differentially methylated area was determined by calculating standard deviation of the methylation values of the 16 control samples. SD value for each individual patient was calculated based on the standard deviation of the controls. A value <-2SD indicates a hypomethylated CpG. A patient was considered *HOXA4* hypomethylated when at least two-thirds of the CpG sites in the differentially methylated area of *HOXA4* were <-2SD.

Data visualization (Study III)

The Integrated Genome Browser (IGB) (Freese et al., 2016) was used to visualize methylation patterns of the genome-wide methylation data and to integrate information such as location of CGIs, genes and TSSs that overlapped with specific CpGs.

Statistical test name	Description	Reference	Number of study in which the test was used	Description of use
Analysis of variance (ANOVA)	A statistical procedure that determines whether any differences exist among two or more groups of subjects on one or more factors	Dawson & Trapp, 2004	III, IV	Comparisons of differences between UPD(7)mat samples and controls in qPCR analysis (III), comparisons of group differences in Epityper data set (IV)
Dunnett T-test/Dunnett's procedure	A multiple comparison method for comparing multiple treatment groups with a single control group following a significant F test in analysis of variance	Dawson & Trapp, 2004	IV	Comparison of the mean level of methylation in subgroups of SRS and SGR patients against a control group (IV)
Kolmogorov-Smirnov	A test for assessing normality of data	Ghasemi & Zahedias,	IV	Normality testing of Illumina data for BAMSE analysis (IV)
Levene's test	A test of the equality of two variances	Dawson & Trapp, 2004	IV	Equal variance testing for Epityper data (IV)
Linear regression analysis	An analysis to study the linear relationship between a dependent variable and one or more independent variables	Schneider et al., 2010	II	Methylation level analysis using different models (II)
Pearson product-moment correlation coefficient	A measure of the linear relationship between two numerical measurements made on the same set of subjects	Dawson & Trapp, 2004	IV	Correlation of methylation and height for normally distributed data (IV)
Shapiro-Wilk	A test for assessing normality of data	Ghasemi & Zahedias, 2012	IV	Normality testing of Epityper data (IV), normality testing of Illumina and EpiTYPER control data (IV), normality testing of expression data (IV)
Spearman rank-order correlation coefficient	A nonparametric correlation that measures the tendency for two measurements to vary together	Dawson & Trapp, 2004	IV	Correlation of methylation and height for data that was not normally distributed (IV), correlation analysis of expression data when data was not normally distributed (IV)
T-test	The statistical test for comparing a mean with a norm or for comparing two means	Dawson & Trapp, 2004	II, III	Comparing characteristics between groups for continuous variables (II), calculation of the difference of means between UPD(7)mat samples and controls in qPCR analysis (III)
$\chi 2$ test	The statistical test used to test the null hypothesis that proportions are equal or, equivalently, that factors or characteristics are independent or not associated	Dawson & Trapp, 2004	II	Comparing characteristics between groups for categorical variables (II)

Table 2. Statistical tests

RESULTS AND DISCUSSION

Study I

Genomic aberrations in SRS

A genome-wide Affymetrix genotyping array was used to study potential genomic aberrations in SRS patients with 11p15 LOM and clinical SRS (unknown etiology). Four of the 12 SRS patients with unknown etiology were termed SRS-like due to fewer fulfilled clinical criteria. None of the patients had UPD(7)mat.

Altogether 60 CNVs were identified with CNAG 2.0 and 687 CNVs by the more sensitive Aroma.Affymetrix program. When combining the data from these two programs, a list of 33 CNVs was obtained that included only CNVs reported by both programs. The largest CNVs were identified in 3 out of 12 patients with clinical SRS: a heterozygous deletion of chromosome 15q26.3 including *IGF1R* (2,6 Mb), a 22q11.21-q11.22 deletion (1,1 Mb) and a pseudoautosomal Xp22.33 duplication (2,7 Mb). All three CNVs were confirmed with qPCR analysis and there were no CNVs reported in DGV (Database of Genomic Variants) that would have entirely covered these regions.

Large CNVs on 14q11.2, 15q11.2 and 17q21.3 occurred in multiple patients, but these regions are known to carry segmental duplications and these CNVs can be considered as normal variation between individuals. A search for CNVs that were found in at least three SRS patients, but had not been reported to DGV, yielded 10 regions containing known genes: *CNTNAP2* (contactin associated protein like 2), *RERG* (RAS like estrogen regulated growth inhibitor), *AGPAT5* (1-acylglycerol-3-phosphate O-acyltransferase 5), *CSRP1* (cysteine and glycine rich protein 1), *ITGB8* (integrin subunit beta 8), *SPOCK1* (SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1), *TFCP2* (transcription factor CP2), *FRMD6* (FERM domain containing 6), *POU6F1* (POU class 6 homeobox 1) and *SH3MD4* (SH3 domain containing ring finger 3).

LOH regions were determined in order to search for regions potentially corresponding to recessive disease alleles. Two regions of LOH, 8p11.21-q11.23 and 13q31-q32, corresponded to regions previously reported in patients with SRS-like features. The 8p11.21-q11.23 LOH found in a SRS patient of unknown etiology overlapped with a deletion of 8q11-q12 reported in a patient with SRS-like features (Schinzel et al., 1994). The 13q31-q32 LOH region in another patient with SRS of unknown etiology corresponded to one reported patient with SRS-like features and a chromosome 13q deletion (Wahlström et al., 1993). The 13q31-q32 LOH regions spans three genes: *GPC5* (glypican 5), *GPC6* (glypican 6) and *SOX21* (SRY-box 21). The glypicans are a family of glycoproteins, which may play a role in the control of cell division and growth regulation. Interestingly, one member of this family, *GPC3*, causes Simpson-Golabi-Behmel overgrowth syndrome (Cottereau et al., 2013).

All of the three largest CNV regions (15q26.3 heterozygous deletion including *IGF1R* (2,6 Mb), a 22q11.21-q11.22 deletion (1,1 Mb) and a pseudoautosomal Xp22.33 duplication (2,7 Mb) correspond to reported cases of growth restriction. 15q deletions including the *IGF1R* gene have been characterized with pre- and postnatal growth restriction, microcephaly, developmental delay, skeletal abnormalities and dysmorphic facial features (Rudaks et al., 2011), while increased dosage of 15q results in overgrowth (Tatton-Brown, 2009). The patient with 15q26.3 deletion had pre- and postnatal growth restriction, developmental delay, triangular face and micrognathia, but had relative macrocephaly instead of the microcephaly that is typical in *IGF2R* deletion patients. The patient with a 22q11.21-q11.22 deletion presented with pre- and postnatal growth restriction, among other features. 22q11.2 deletion syndrome, with a heterogeneous clinical presentation, is associated with intrauterine growth restriction in 4% of the patients and short stature in 15% of the patients (McDonald-McGinn et al., 2015). The patient with pseudoautosomal Xp22.33 duplication including the short stature homeobox (*SHOX*) gene presented with both pre- and postnatal growth restriction and had no response to GH treatment. Although *SHOX* mutations and deletions are typically associated with short stature, also *SHOX* duplications have been described in patients with idiopathic

short stature (ISS), including a report of six ISS patients with *SHOX* duplications (Benito-Sanz, 2011).

Study II

Methylation changes in individuals born preterm

CpGs of the *IGF2* gene were studied in adults born preterm at very low birth weight. These individuals had elevated levels of cardiovascular disease risk factors in comparison to controls born at term. Individuals who are born preterm at very low birth weight have a higher risk for adult-onset disease, and these risks might be carried through epigenetic changes of imprinted genes. The aim of Study I was to test for the presence of epigenetic changes in *IGF2*. We found that the difference in methylation level of one CpG in *IGF2* gene was statistically significant between individuals born preterm at VLBW and controls, 20 years after birth. The difference was significant after correction for multiple variables that could have affected the results, such as plate number, sex, age, BMI, mother's smoking during pregnancy, age of parents, mother's BMI before pregnancy and highest education of either parent. The difference was around 2%, and also the other sites in the same area had a similar trend showing lower methylation in the subjects compared to controls. The results were similar to the Dutch famine study (Heijmans et al., 2008), where people exposed to early nutritional deprivation had a 5% decrease in methylation level of the same region in IGF2, measured in adulthood. The results were significant for the same CpGs in both preterm infants who were appropriate for gestational age (AGA) and SGA.

Study III

Genome-wide analysis of methylation data in UPD(7)

The aim of Study III was to use a genome-wide methylation assay to study the methylation differences between UPD(7)mat patients, controls and a rare

UPD(7)pat sample in order to identify new differentially methylated regions (DMRs) and imprinted genes on chromosome 7. Since maternal and paternal alleles of imprinted genes are differentially methylated at DMRs, comparing UPD(7)mat and UPD(7)pat samples with controls should indicate regions where uniparental disomies cause significantly higher or lower levels of methylation compared to control samples. Initial genome-wide analysis indicated that the significantly differentially methylated regions (genome-wide significance threshold, $p=5 \times 10^{-8}$) between UPD(7)mat patients and controls were located exclusively on chromosome 7, as expected. A similar pattern was observed in differentially methylated CpGs between UPD(7)pat vs. controls clustering on chromosome 7. These results confirmed that the approach was useful in highlighting genomic locations where DMRs were affected by uniparental disomy, and the study then proceeded with targeting chromosome 7 only.

Differentially methylated regions on chromosome 7

The analysis of chromosome 7 included 30,017 CpG sites. The filtering approach was able to pinpoint DMRs of already known imprinted genes on chromosome 7; *GRB10* (7p12.2), *SGCE/PEG10* (sarcoglycan epsilon/paternally expressed 10, at 7q21.3) and *MEST/MESTIT1* (mesoderm specific transcript/MEST intronic transcript 1, antisense RNA, at 7q32), confirming that the approach was useful in finding DMRs of imprinted genes. DMRs were also found for the two previously predicted imprinted genes, *HOXA4* (7p15.2) and *GLI3* (GLI family zinc finger 3, at 7p13), as well as for *PON1* (paraoxonase 1, at 7q21.3), a disputed imprinted gene. Other found DMRs were located near eight genes [*MAD1L1* (mitotic arrest deficient 1 like 1, at 7p22), *CARD11* (caspase recruitment domain family member 11, at 7p22), *RPS2P32* (ribosomal protein S2 pseudogene, at 7p15.3), *PRR15* (proline rich 15, at 7p14.3), *SH2B2* (SH2B adaptor protein 2, at 7q22), *SVOPL* (SVOP like, at 7q34), *HTR5A* (5-hydroxytryptamine receptor 5A, at 7q36.1) and *RARRES2* (retinoic acid receptor responder 2, at 7q36.1] and two intergenic regions with lncRNAs.

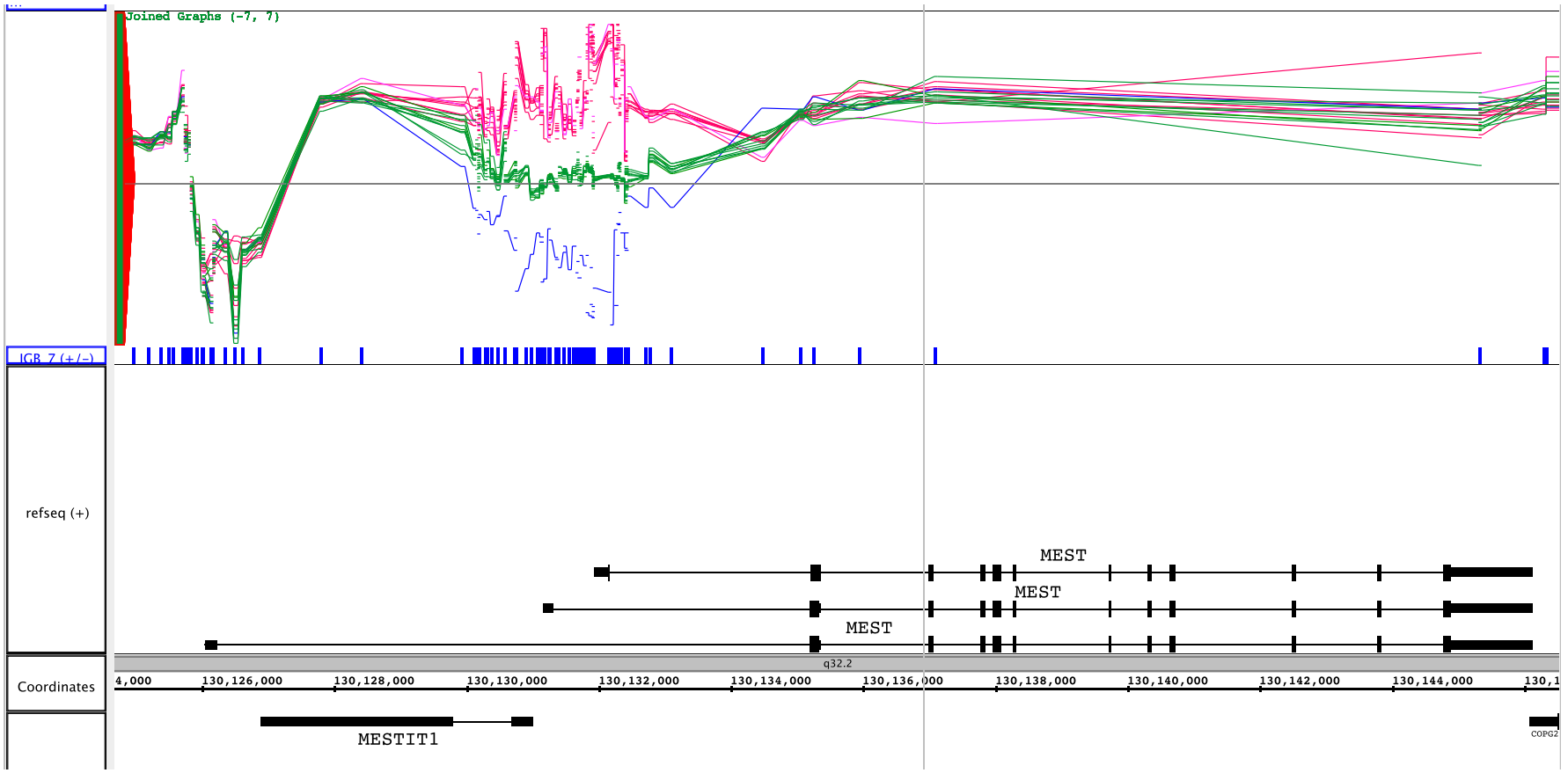


Figure 7 a. Known DMR of MEST-MESTIT1 at 7q32, the DMR with the highest number of differentially methylated CpGs (63) found in study III. Methylation level of UPD(7)mat represented in red lines, controls in green and UPD(7)pat in blue. The image is a copy of the IGB output.

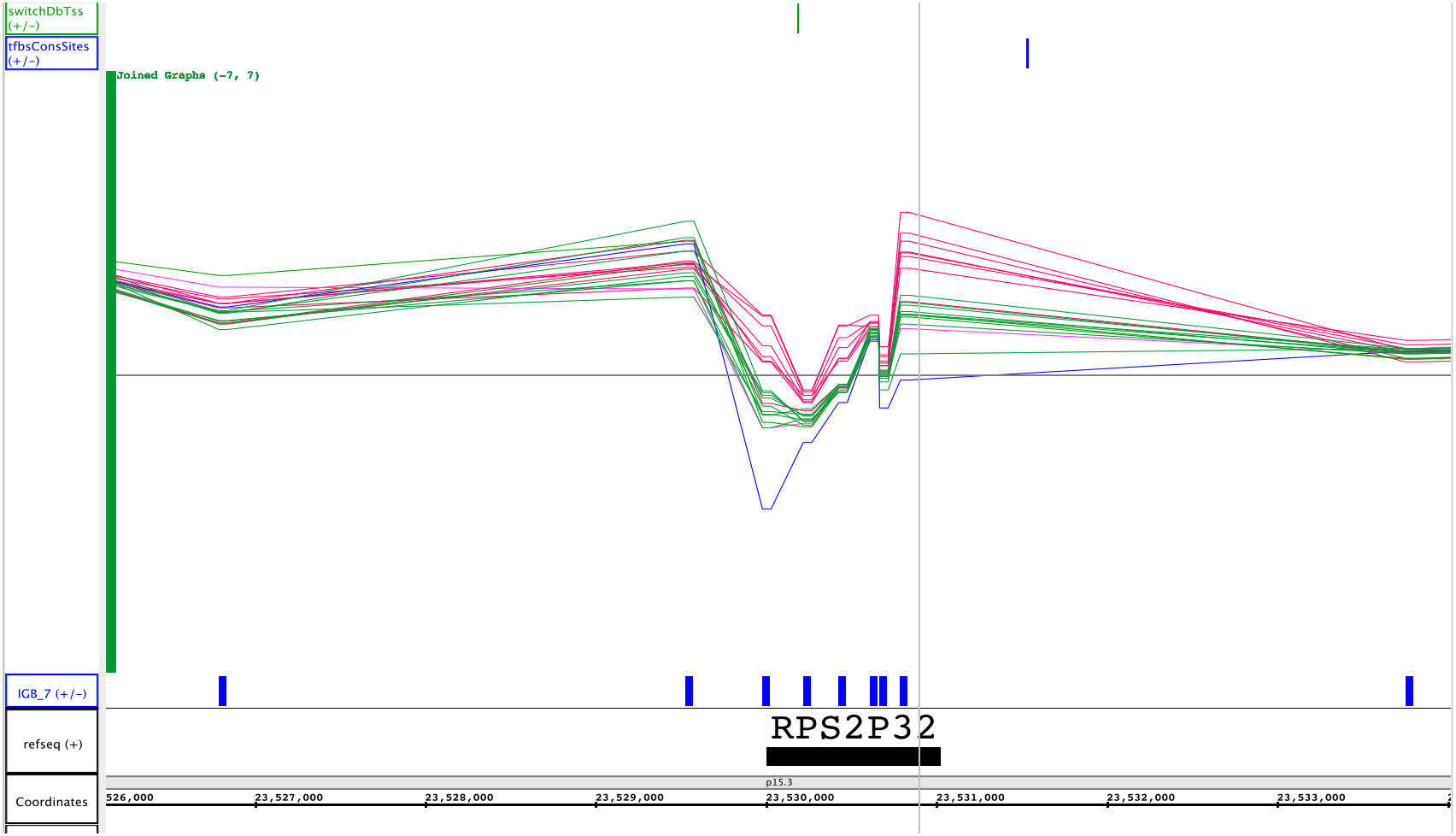


Figure 7 b. Newly identified DMR of RPS2P32 at 7p15.3. Methylation level of UPD(7)mat represented in red lines, controls in green and UPD(7)pat in blue. The image is a copy of the IGB output.

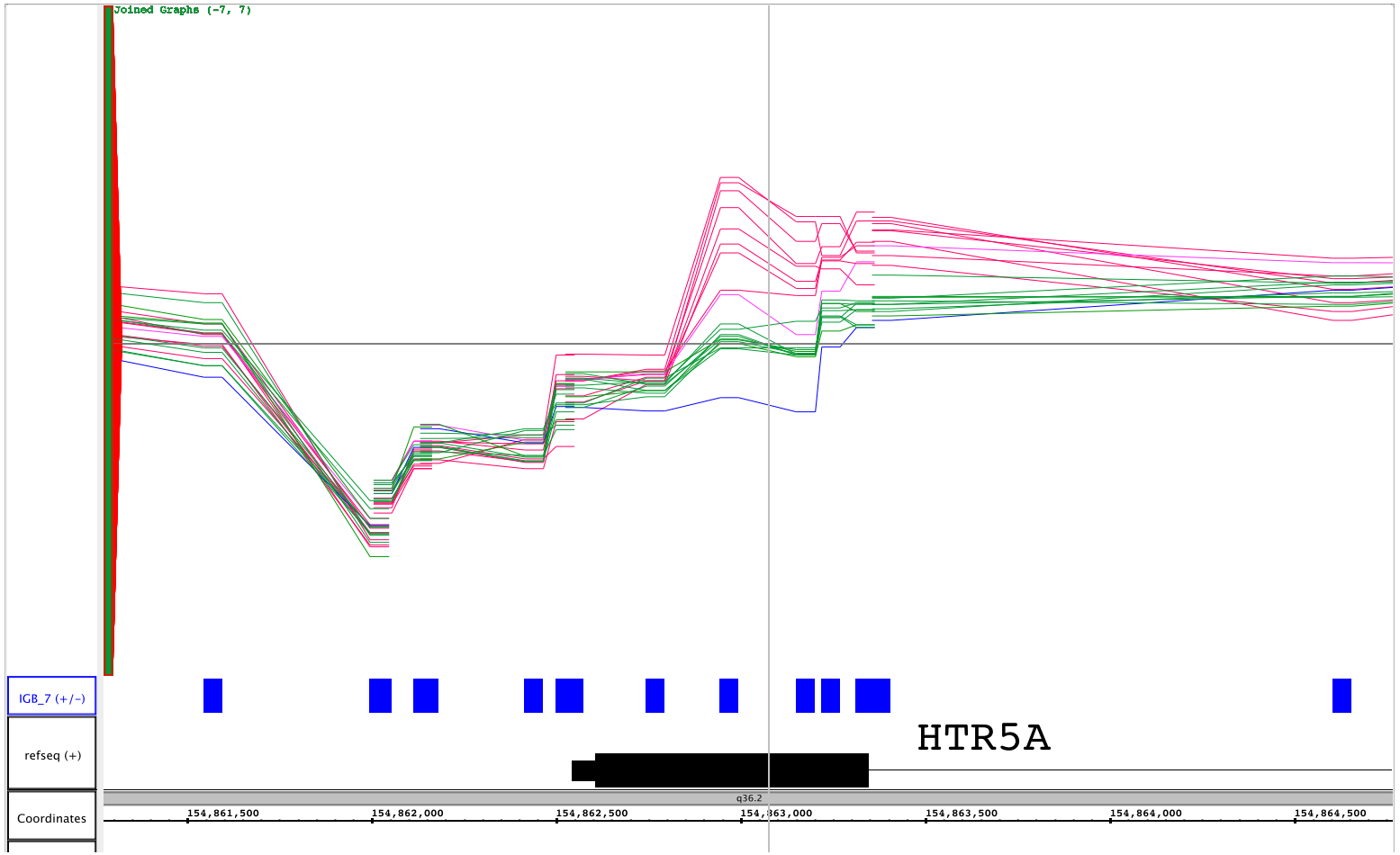


Figure 7 c. Newly identified DMR of HTR5A at 7q36.1. Methylation level of UPD(7)mat represented in red lines, controls in green and UPD(7)pat in blue. The image is a copy of the IGB output.

Validation of methylation status with pyrosequencing

In order to test whether the methylation results could be replicated by using another methylation assay, CpG sites of *HTR5A* and *HOXA4* were studied by pyrosequencing. There was a high correlation (94-95%) of methylation levels of corresponding CpG sites obtained by the Infinium HumanMethylation450K BeadChip and pyrosequencing, indicating reliability of the results.

Differentially expressed genes on chromosome 7

After detecting DMRs, qPCR was used in order to verify possible imprinted expression in blood. The known imprinted gene *PEG10* was used as a reference for imprinted expression and a statistically significant (p=0.0017) difference was observed between expression level of *PEG10* in UPD(7)mat and controls. *PEG10* also showed a pattern of expression that would be expected for an imprinted gene; average expression of controls was in the middle and UPD(7)mat and UPD(7)pat deviated in opposite directions. *HOXA4*, *GLI3* and *SVOPL* showed a statistically significant difference between expression level of UPD(7)mat and controls (p-values 0.008, 0.049 and 0.017, respectively). Also the general pattern of expression was similar to *PEG10*. For some of the studied genes, a pattern suggestive of imprinted expression was observed, but the differences between UPD(7)mat and controls were not statistically significant. For others, expression levels did not suggest imprinting. For *HTR5A* and *PON1*, expression in blood was not detected, so it was not possible to carry out further studies. Expression levels of genes located close to the intergenic regions with detected DMRs were not studied.

Parent-of-origin allele-specific expression analysis

Allele-specific expression analysis was conducted in order to confirm parent-of-origin specific expression. Exonic SNPs of parent-child trios of the tree genes with differential expression; *HOXA4*, *SVOPL* and *GLI3,* were studied. In order to determine expression from a single parental allele, it was required to find exonic SNPs for which the child is heterozygous and preferably both parents homozygous for a different allele. For *SVOPL*, two trios were found and both of them showed expression of only the maternal allele (Figure 8). This confirmed

maternal expression of *SVOPL*, as suggested by the previous expression results of UPD(7)mat compared to controls. For *HOXA4*, one heterozygous SNP was found and cDNA sequencing confirmed monoallelic expression. As both parents were heterozygous, it was not possible to determine which parental allele was expressed, but monoallelic expression supports imprinted status of the gene. For *GLI3*, conclusive results were not obtained due to technical difficulties with PCR.

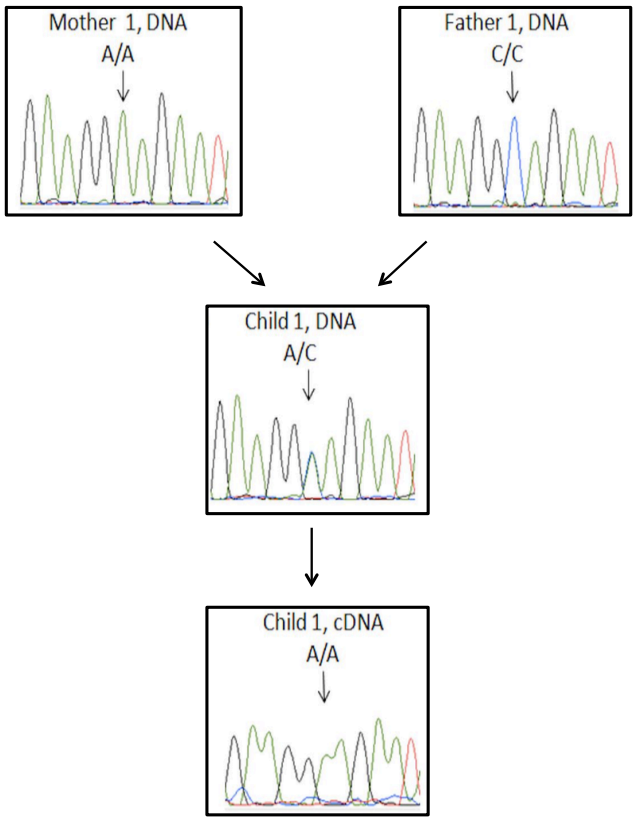


Figure 8. SNP rs2305816 at *SVOPL* for a parent-child trio. Both mother and father are homozygous for different alleles, the child is therefore heterozygous A/C, but only the mother's allele A is expressed in the child. Modified from Hannula-Jouppi et al., 2014.

Taken together, 10 novel DMRs were found in chromosome 7, two DMRs for the predicted imprinted genes *HOXA4* and *GLI3* and one DMR for a disputed imprinted gene *PON1*. Differential expression was confirmed for *HOXA4, GLI3*

and *SVOPL* in blood. *SVOPL* was further found to be expressed only from the maternal chromosome by allele-specific parent-of-origin expression analysis. *HOXA4* was found to have monoallelic expression, supporting its imprinted status.

HOXA4 belongs to a cluster of homeobox-containing transcription factors that control the anterior-posterior patterning during development (Lewis, 1992). *GLI3* is a transcription factor that affects tissue patterning and its defects cause multiple anomalies especially involving the digits (Crapster et al., 2017). As for *SVOPL*, methylation changes have been reported in pseudohypoparathyroidism patients with multi-locus imprinting defects (Rochtus et al., 2016). The observed changes in the expression levels of these genes in UPD(7)mat patients may have functional consequences affecting the phenotype of the patients.

Study IV

Common epigenetic changes in different SRS subgroups

The objective of Study IV was to use a genome-wide methylation array to look for common DNA methylation changes shared among the three subgroups of SRS [UPD(7)mat, 11p15 LOM and clinical SRS]. Although there are differences between the clinical characteristics of the three subgroups, major features, such as growth restriction, are shared by the patients. It was hypothesized that the subgroups may share common DNA methylation changes that could be found using a genome-wide array. As DMRs of imprinted genes often involve longer stretches of CpGs, the strategy was to devise a filtering system that would allow identification of longer stretches of CpGs.

Stretches of consecutive CpGs were found differentially methylated in all SRS subgroups in 26 genes or intergenic loci on 13 different chromosomes. For most of these loci, the median methylation differences between controls and

subgroups were small (less than 2%). Only two separate loci included CpG sites with median methylation differences greater than 5% between controls and all three subgroups, an intergenic region at 5p15.33 and *HOXA4*. The region at *HOXA4* included altogether 12 consecutive CpG sites with significant methylation differences between controls and all of the subgroups, and an additional stretch of 3 CpGs with similar differences. *HOXA4* was therefore selected as a clear candidate for further study.

HOXA4 **CpG ID**	**11p15 LOM**	**Clinical SRS**	**UPD(7)mat**
cg02022102	0,1337	0,3379	0,0345
cg03982801	0,4355	0,2119	0,8381
cg02706018	0,9538	0,3056	0,5738
cg09574499	0,8489	0,2095	0,4154
cg14042889	0,6536	0,8850	0,6749
cg11532431	**0,0011**	**0,0013**	**8,2E-06**
cg00562553	**0,0052**	**0,0037**	**0,0002**
cg22997113	**0,0006**	**0,0001**	**1,5E-06**
cg04317399	**0,0309**	**0,0030**	**4,1E-05**
cg07317062	**0,0073**	**0,0014**	**8,4E-06**
cg19142026	**0,0061**	**0,0005**	**7,3E-06**
cg11410718	**0,0037**	**0,0004**	**3,3E-06**
cg17457637	**0,0349**	**0,0011**	**1,4E-05**
cg06942814	**0,0116**	**0,0004**	**1,9E-05**
cg08657492	**0,0063**	**0,0001**	**4,4E-06**
cg04321618	**0,0172**	**0,0009**	**9,7E-06**
cg14359292	**0,0125**	**0,0001**	**9,8E-07**
cg25952581	0,0818	0,0125	0,0002
cg24169822	**0,0120**	**0,0005**	**5,4E-06**
cg17591595	**0,0371**	**0,0006**	**0,0033**
cg11908057	**0,0108**	**0,0001**	**0,0031**
cg25967031	0,0804	0,0035	0,0167
cg15196806	0,0526	0,0173	0,0674
cg15624376	0,8751	0,8147	0,2402
cg20161965	0,0634	0,2399	0,0240

Table 3. P-values for differential methylation between each of the SRS subgroups and controls for all *HOXA4* CpGs of the Illumina 450K BeadChip assay that passed quality control. P-values <0,05 are highlighted in red. Modified from Muurinen et al. 2017.

For most of the CpGs, the hypomethylation of *HOXA4* was strongest for UPD(7)mat patients and weakest for 11p15 patients. UPD(7)mat itself may explain the methylation differences seen in the patients, although there was one

UPD(7)mat patient who was not at all hypomethylated at *HOXA4* and another UPD(7)mat patient with hypomethylation only at three *HOXA4* sites. The hypomethylation of 11p15 LOM patients may be explained by multi-locus imprinting disturbance (MLID).

Localization of the CpG with lowest methylation levels in subgroups relative to the controls was close (within 5-17 bp) to the TSS, which suggested that the found methylation change might have relevance in controlling expression level of *HOXA4*.

	Group/patient	Average birth length SD	Average birth weight SD	Average postnatal height in SD (measurement closest to 24 months)
SRS	11p15 LOM	-4,9	-4,0	-4,4
	clinical SRS	-3,8	-3,4	-3,6
	UPD(7)mat	-3,4	-2,9	-4,8
	UPD(7q21-qter)mat	-5,2	-4,5	-3,0
	SGR	-3,3	-2,6	-3,8

Table 4. Average height and weight data of SRS and SGR patients in Study IV.

Targeted study of *HOXA4* with EpiTYPER

EpiTYPER assay was used for a targeted study of the methylation of *HOXA4* promoter region. One EpiTYPER CpG site, cg04317399 at chr7:27170313, was also covered by the Illumina 450K array and showed a strong correlation (R^2=0.86) between the two methods. Additional three CpG sites covered by EpiTYPER showed similar results for the SRS patients as the 450K study, validating the previous results of *HOXA4* hypomethylation at the promoter region in SRS patients.

Additionally, 39 severely growth restricted children were included in a targeted study to find out whether *HOXA4* hypomethylation could be present in children with SGR. The results revealed that similarly to all SRS subgroups, the SGR

patients were significantly differentially methylated compared to controls at all four studied sites. Analysis of individual patients showed that 44% of the SGR patients were hypomethylated in at least two-thirds of the probes at *HOXA4* promoter region. The largest difference (I-J=-0.249, $p = 3.389 \times 10^{-7}$) between subjects and controls was found for clinical SRS (I) compared to controls (J). The targeted study therefore revealed that *HOXA4* hypomethylation was not limited to SRS patients but was also present in the SGR patients.

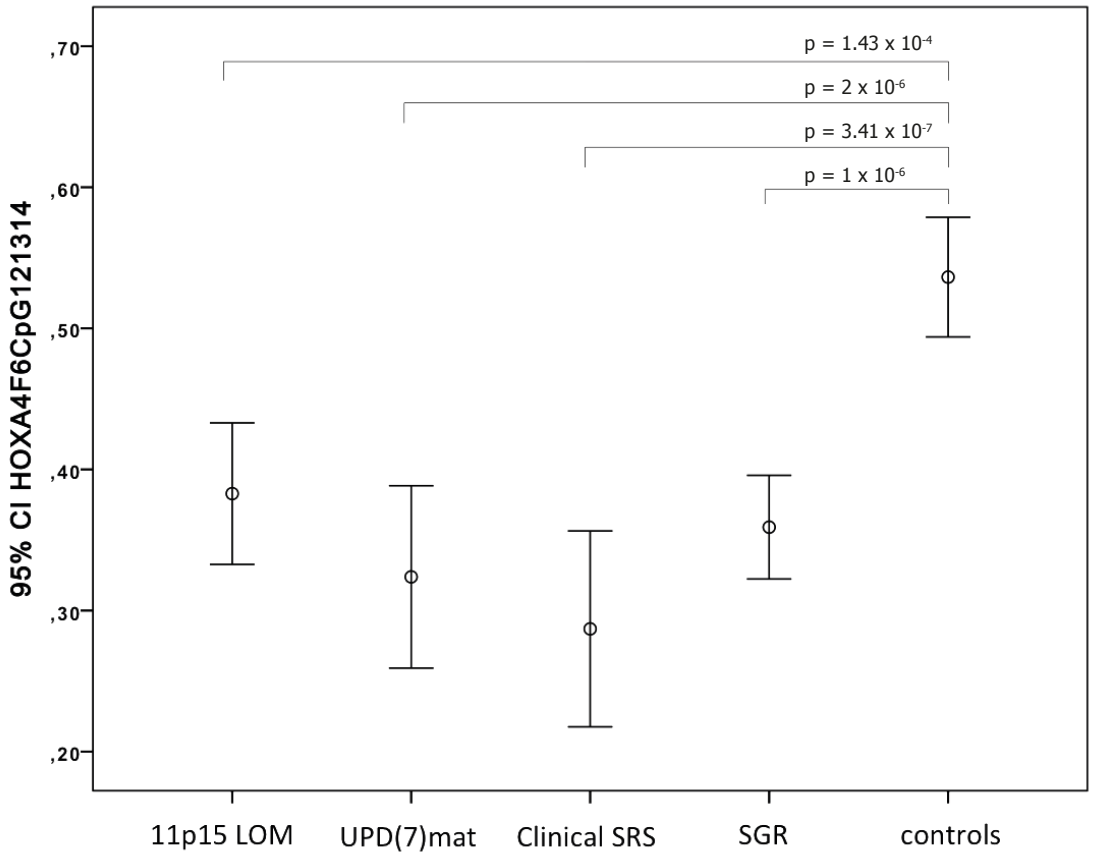


Figure 9. Methylation levels measured at EpiTYPER CpG unit HOXA4F6CpG12.13.14 with the largest difference between clinical SRS patients and controls. Modified from Muurinen et al., 2017.

Frequency of 11p15 LOM higher than previously tested

During preliminary analysis of the 450K methylation study, it became evident that more patients had 11p15 LOM than had been previously identified with a restriction site-specific methylation method (Bruce et al., 2008). An area with 36 CpG sites was tested and a patient was considered hypomethylated when at least two-thirds of the CpGs were hypomethylated (below -2 SD of the controls). The results were similar to those published in another genome-wide methylation study, where Infinium Human Methylation 450K BeadChip array detected more SRS patients with 11p15 LOM than methylation-sensitive RFLP (restriction fragment length polymorphism) PCR method (Prickett et al. 2015). Genome-wide arrays may therefore be more sensitive at detecting methylation changes than some of the other methods.

Correlation of *HOXA4* methylation level with height in healthy children

Since *HOXA4* promoter hypomethylation was present in both SRS and SGR patients, the next step was to study whether *HOXA4* methylation level was associated with height in healthy children. Illumina 450K BeadChip methylation data from 227 children was used to study the correlation of methylation level of *HOXA4* promoter with relative height at school age. Both height measurements and DNA samples for methylation testing had been taken at approximately 8 years of age. In order to make the height measurements comparable to each other, the height of each individual was converted to SD scale. The results showed that the height of the children correlated with methylation level at multiple sites of the *HOXA4* promoter region, for example at cg11908057 at age eight ($r=0.148$, $n=225$, $p=0.026$). Next, the correlation between height at age eight and genotypes of some the most strongly height-associated SNPs were studied in the same individuals. No significant association was found between height and genotype. Therefore, in the dataset of healthy children, the methylation level of cg11908057 at *HOXA4* was better at explaining height variation at school-age than some of the best height-associated SNPs. Interestingly, cg11908057 is located in a potential regulatory protein-binding site for an enhancer of zeste homolog 2 (*EZH2*), which is a methyltransferase enzyme encoded by the *EZH2* gene on 7q36.1. *EZH2* is implicated in Weaver

syndrome, a rare congenital syndrome characterized by pre- and postnatal overgrowth and tall adult stature (Gibson et al., 2012)

Correlation of methylation level of *HOXA4* promoter with expression of *HOXA4*

As the hypomethylated area of *HOXA4* was located at the promoter region, the next step was to study whether methylation level of the promoter region correlated with the expression level of the *HOXA4* gene in blood. As expression level of *HOXA4* had already been measured in Study III to verify imprinted expression, it was possible to use the same data for Study IV in order to study the correlation between methylation level of *HOXA4* promoter and expression in UPD(7)mat, UPD(7)pat and controls. Statistically significant negative correlation was observed between methylation level and expression in all differentially methylated *HOXA4* CpGs. cg22997113 showed the strongest negative correlation ($r=-0.81$, $n=20$, $p=1.40 \times 10^{-5}$). The results suggested that DNA methylation level at the promoter of *HOXA4* may regulate *HOXA4* expression.

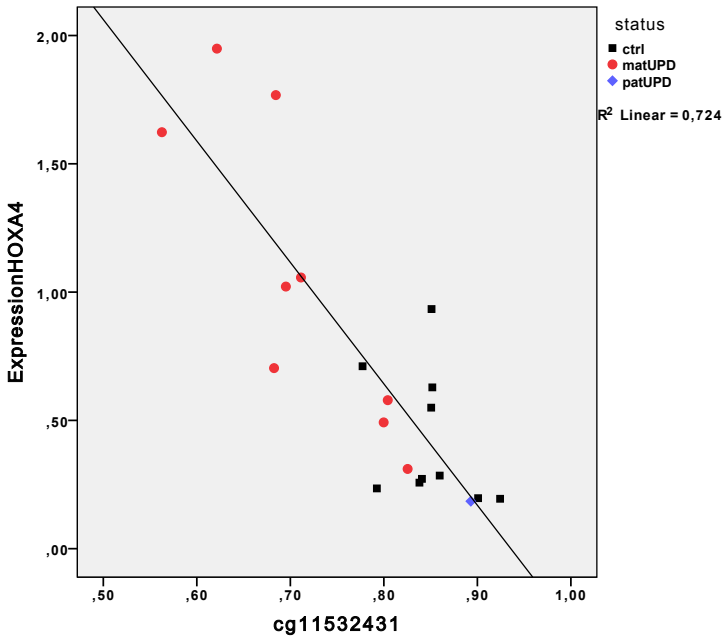


Figure 10. *HOXA4* expression level in blood vs. methylation level of *HOXA4* cg11532431.

Taken together, this study demonstrated a methylation change shared by UPD(7)mat, 11p15 LOM and clinical SRS patients. The *HOXA4* promoter region hypomethylation was present in 55% of the SRS patients and targeted studies of the region revealed that it was also present in 44% of patients with SGR. A statistically significant negative correlation was demonstrated between *HOXA4* expression and promoter methylation level in blood. In further analysis, it was found that *HOXA4* promoter methylation level was correlated with height in healthy children and the effect size was larger than for any of the nine tested highly significant height-associated SNPs.

The *HOXA4* gene is located on 7p15.2, in a cluster of *HOXA* genes. Multiple *HOXA* genes have been predicted to be maternally expressed imprinted genes (Luedi et al., 2007). *HOX* genes are also highly conserved across species. Mouse *Hoxa4* loss-of-function homozygous mutants have skeletal abnormalities including partial anterior transformation of C3, posterior transformations of C7 to T1, presence of C7 cervical rib and malformation of the sternum (Horan et al., 1994)(Kostic & Capecchi, 1994). Mutations of the human *HOX* genes cause phenotypes such as facial dysmorphisms, various limb and digit anomalies, cardiac defects and urogenital malformations (Quinonez & Innis, 2014). The known functions of *HOX* and *Hoxa4* genes suggest that altered expression levels of *HOXA4* may also have relevance regarding skeletal malformations in SRS. Upregulation of several *HOX* genes has also been reported in 3-M syndrome (Murray et al., 2013), a growth restriction syndrome with a similar phenotype to SRS. An association between birth weight and methylation level of two CpGs has been observed in *HOXA3*, the gene adjacent to *HOXA4* (Simpkin et al., 2015), demonstrating that the region may play a role in regulating body size.

Limitations of the studies

The studies in this thesis utilize genome-wide approaches in order to search for new variants contributing to SRS and growth restriction. Both genotyping arrays and genome-wide methylation arrays are limited by the number of probes they contain and the array design that determines the distribution of the probes across the genome. Therefore some true genetic/epigenetic changes are probably left undetected by these studies. Quality control steps further reduce the number of informative sites for analysis, such as the reduction of CpG sites from over 450 000 to 361 950 after quality control in Study IV.

The genome-wide methylation studies are especially affected by the design of the filtering approach. It is possible that single CpG sites on the array would have been relevant, but in the approaches used in Study III and Study IV, they were filtered out. Study IV is also affected by the chosen approach to look for methylation changes common to all SRS subgroups. Another type of approach, targeting single groups or cases, would have given very different results.

Sample size is a common limiting factor in studies of rare diseases, such as SRS. Limited sample size was especially apparent in Study III, with only eight UPD(7)mat samples, one UPD(7q31-qter)mat sample and one UPD(7)pat sample. However, these extremely rare samples enabled the study of DMRs of chromosome 7 in a unique way, and the small sample size is therefore justified. There are only a handful of UPD(7)pat cases reported in literature, and obtaining more samples would not have been possible.

In Study IV, 16 control samples were used to calculate normal levels of methylation that was then compared to the methylation level of the patients. A larger number of control samples would have improved the accuracy of establishing a normal methylation level.

Study II would have also benefited from a larger sample size. Although the results were interesting and concordant with other studies, it was not possible to

assess whether a 2% methylation level difference in a CpG site of *IGF2* would have clinical relevance.

Methylation levels of imprinted loci have been shown to vary between tissues of a single individual. The studies in this thesis are limited to observations of methylation levels in blood and may not reflect the status in other tissues that may be more relevant for growth.

CONCLUSIONS AND FUTURE PROSPECTS

Studies I, III and IV demonstrated the usefulness of genome-wide arrays in both genetic and epigenetic studies of a rare imprinting disorder. Due to the heterogeneous etiology of SRS, untargeted genome-wide arrays are especially useful in finding molecular aberrations for single patients (Study I) as well as groups of patients (Study IV). Genome-wide methylation arrays can also be tailored for specific purposes, such as finding certain types of DNA methylation patterns by creating filtering systems (Studies III and IV).

Atypical clinical presentation in syndromic patients, such as the SRS-like patients in Study I, complicates making a diagnosis and therefore targeted studies may not always lead to finding the underlying molecular abnormality. In such cases, genome-wide arrays may prove to be especially useful, and may lead to detection of an alternative diagnosis. An example of this was the finding of an *IGF2R* deletion in a SRS-like patient (Study I), who presented with relative macrocephaly, a clinical feature very typical of SRS patients, instead of microcephaly that is usually seen in *IGF2R* deletion patients.

Imprinted genes, in particular, may be sensitive to environmental influences and the association between epigenetic changes of imprinted genes and environmental influences has been demonstrated in many studies. Study II and the Dutch famine study both demonstrate lower methylation levels of *IGF2* CpG sites in individuals with early adverse events (VLBW pre-term born individuals in Study II and early caloric restriction in the Dutch famine study) and later risk of adult-onset disease. Altered methylation level at *IGF2* therefore might provide a potential mechanism for later disease risk development. This mechanism for the origin of adult-onset disease may be confirmed in future studies.

The causative imprinted genes on chromosome 7 for SRS in UPD(7)mat patients are not known. Study III was able to provide a picture of the methylation landscape of chromosome 7 in regions where methylation levels are affected by parental origin. These regions may indicate regions potentially responsible for

the SRS phenotype. In addition to differences in methylation, *HOXA4, GLI3* and *SVOPL* were found to have expression differences, suggesting imprinted expression of these genes in blood. Lack of differential expression for some of the other genes in blood does not rule out imprinting in other tissues. Parent-of-origin specific expression analysis also confirmed *SVOPL* as a maternally expressed imprinted gene. However, little is known about the function of *SVOPL. HOXA4* and *GLI3* were both interesting findings of the study as they have been shown to have important roles in body patterning during development.

As growth restriction is one of the key features shared by all subgroups of SRS, Study IV aimed to explore whether a common epigenetic change would be shared by the SRS subgroups. A clear region with multiple adjacent hypomethylated CpGs was found in the promoter region of *HOXA4*. This region was remarkably larger in size and with greater methylation differences than any other region in the genome-wide screen. *HOXA4* hypomethylation was found not to be specific for SRS, but was also found in a substantial proportion of patients with severe growth restriction of unknown etiology. A further study found a correlation between *HOXA4* methylation level and height of healthy school-aged children. Study IV led to a hypothesis that *HOXA4* may play a role in regulating pathways relevant for SRS, growth restriction and height in general. The study suggests a new target region for future studies in SRS, imprinting and growth. A better understanding of the molecular mechanisms that influence SRS will eventually lead to better diagnostics, counseling and treatment for the patients.

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REFERENCES:

Abu-Amero, S., Monk, D., Frost, J., Preece, M., Stanier, P. & Moore, G. E. (2008). The genetic aetiology of Silver-Russell syndrome. *J. Med. Genet*. 45(4), 193-9.

Adkins, R. M., Somes, G., Morrison, J. C., Hill, J. B., Watson, E. M., Magann, E. F. & Krushkal, J. (2010). Association of Birth Weight With Polymorphisms in the IGF2, H19, and IGF2R Genes. *Pediatr. Res.* 68(5), 429-34.

Akawi, N. A., Ali, B. R., Hamamy, H., Al-Hadidy, A. & Al-Gazali, L. (2011). Is autosomal recessive Silver-Russell syndrome a separate entity or is it part of the 3-M syndrome spectrum? *Am. J. Med. Genet. A.* 155A(6), 1236-45.

Allen, C. & Reardon, W. (2005). Assisted reproduction technology and defects of genomic imprinting. *BJOG*. 112(12), 1589-94.

Aryee, M. J., Jaffe, A. E., Corrada-Bravo, H., Ladd-Acosta, C., Feinberg, A. P., Hansen, K. D. & Irizarry, R. A. (2014). Minfi: A flexible and comprehensive Bioconductor package for the analysis of Infinium DNA Methylation microarrays. *Bioinformatics.* 30(10), 1363-1369.

Azzi, S., Blaise, A., Steunou, V., Harbison, M. D., Salem, J., Brioude, F., Rossignol, S., Habib, W. A., Thibauld, N. Neves, C. D., Jule, M. L., Brachet, C., Heinrichs, C., Bouc, Y. L. & Netchine, I. (2014). Complex tissue-specific epigenotypes in Russell-Silver Syndrome associated with 11p15 ICR1 hypomethylation. *Hum. Mutat*. 35(10), 1211-20.

Azzi, S., Rossignol, S., Steunou, V., Sas, T., Thibaud, N., Danton, F., Le Jule, M., Heinrichs, C., Cabrol, S., Gicquel, C., Le Bouc, Y. & Netchine, I. (2009). Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. *Hum. Molec. Genet.* 18(24), 4724-4733.

Azzi, S., Salem, J., Thibauld, N., Chantot-Bastaraud, S., Lieber, E., Netchine, I. & Harbison, M.D. (2015). A Prospective study validating a clinical scoring system and demonstrating phenotypical-genotypical correlations in Silver-Russell syndrome. *J. Med. Genet.* 52(7), 446-53.

Babak, T., DeVeale, B., Tsang, E. K., Zhou, Y., Li, X., Smith, K. S., Kukurba K. R., Zhang, R., Li, B., van der Kooy, D., Montgomery, S. B. & Fraser, H. B. (2015). Genetic conflict reflected in tissue-specific maps of genomic imprinting in human and mouse. *Nat. Genet.* 47(5), 544-9.

Ballard, L. M., Jenkinson, E., Byrne, C. D., Child, J. C., Davies, J. H., Inskip, H., Lokulo-Sodipe, O., Mackay, D. J. G., Wakeling, E. L., Temple, I. K. & Fenwick, A. Lived experience of Silver-Russell syndrome: implications for management during childhood and into adulthood. *Arch. Dis. Child*. doi:10.1136/archdischild-2018-314952.

Barker, D.J., Winter, P.D., Osmond, C., Margetts, B. & Simmonds, S.J. (1989). Weight in infancy and death from ischaemic heart disease. *Lancet.* 2(8663), 577–580.

Barlow, D. P. & Bartolomei, M. S. (2014). Genomic Imprinting in Mammals. *Cold Spring Harb. Perspect. Biol.* 6(2), a018382.

Barlow, D. P., Stöger, R., Hermann, B. G., Saito, K. & Schweifer, N. (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature*. 349(6304), 84-7.

Bartholdi, D., Krajewska-Walasek, M., Ounap, K., Gaspar, H., Chrzanowska, K. H., Ilyana, H., Kayserili, H., Lurie, I. W., Schinzel, A. & Baumer, A. (2009). Epigenetic mutations of the imprinted IGF2-H19 domain in Silver-Russell syndrome (SRS): results from a large cohort of patients with SRS and SRS-like phenotypes. *J. Med. Genet.* 46(3), 192-7.

Bartolomei, M. S. & Ferguson-Smith, A. C. (2011). Mammalian genomic imprinting. *Cold Spring Harb. Perspect. Biol*. 3(7).

Bartolomei, M. S., Zemel, S. & Tilghman, S. M. (1991). Parental imprinting of the mouse H19 gene. *Nature*. 351(6322), 153-5.

Barton, S. C., Surani, M. A. & Norris M. L. (1984). Role of paternal and maternal genomes in mouse development. *Nature*. 311(5984), 374-6.

Begemann, M., Spengler, S., Gogiel, M., Grasshoff, U., Bonin, M., Betz, R. C., Dufke, A., Spier, I. & Eggerman, T. (2012). Clinical significance of copy number variations in the 11p15.5 imprinting control regions: new cases and review of the literature. *J. Med. Genet.* 49(9), 547-553.

Begemann, M., Spengler, S., Kanber, D., Haake, A., Baudis., M, Leisten, I., Binder, G., Markus, S., Rupprecht, T., Segerer, H, Fricke-Otto, S. Mühlenberg, R., Siebert, R., Buiting, K. & Eggermann, T. (2011). Silver-Russell patients showing a broad range of ICR1 and ICR2 hypomethylation in different tissues. *Clin. Genet.* (80)1, 83-8.

Begemann, M., Zirn, B., Santen, G., Wirthgen, E., Soellner, L., Büttel, H. M., Schweizer, R., van Workum, W., Binder, G. & Eggermann, T. (2015). Paternally inherited IGF2 mutation and growth restriction. *N. Engl. J. Med.* 373(4), 349-56.

Bengtsson, H., Irizarry, R., Carvalho, B. & Speed, T. P. (2008). Estimation and assessment of raw copy numbers at the single locus level. *Bioinformatics.* 24, 759-67.

Benito-Sanz, S., Barroso, E., Heine-Suner, D., Hisado-Oliva, A., Romanelli, V., Rosell, J., Aragones, A., Caimari, M., Argente, J., Ross, J. L., Zinn, A. R., Gracia, R., Lapunzina, P., Campos-Barros, A. & Heath, K. E. (2011). Clinical and molecular evaluation of SHOX/PAR1 duplications in Leri-Weill dyschondrosteosis (LWD) and idiopathic short stature (ISS). *J. Clin. Endocrinol. Metab.* 96(2), E404-12.

Benonisdottir, S., Oddsson, A., Helgason, A., Kristjansson, R. P., Sveinbjornsson, G., Oskarsdottir, A., Thorleifsson, G., Davidsson, O. B., Arnadottir, G. A., Sulem, G., Jensson, B. O., Holm, H., Alexandersson, K. F., Tryggvadottir, L., Walters, G. B., Gudjonsson, S. A., Ward, L. D., Sigurdsson, J. K., Iodache, P. D., Frigge, M. L., Rafnar, T., Kong, A., Masson, G., Helgason, H., Thorsteinsdottir, U., Gudbjartsson, D. F., Sulem, P. & Stefansson, K. (2016). Epigenetic and genetic components of height regulation. *Nat. Commun*. 7, 13490.

Bibikova, M, Barnes, B., Tsan, C., Ho, V., Klotzle, B., Le, J. M., Delano, D., Zhang, L., Schroth, G. P., Gunderson, K. L., Fan, J. B. & Shen, R. (2011). High density DNA methylation array with single CpG site resolution. *Genomics.* 98(4), 288-95.

Binder, G., Seidel, A-K., Martin, D. D., Schweizer, R., Schwarze, C. P., Wollmann, H. A., Eggermann, T. & Ranke, M. B. (2008). The endocrine phenotype in Silver-Russell syndrome is defined by the underlying epigenetic alteration. *J. Clin. Endocrinol. Metab.* 93(4), 1402-1407.

Binder, G., Seidel, A. K., Weber, K., Haase, M., Wollmann, H. A., Ranke, M. B. & Eggermann, T. (2006). IGF-II serum levels are normal in children with Silver-Russell syndrome who frequently carry epimutations at the IGF2 locus. *J. Clin. Endocrinol. Metab.* 91(11), 4709-4712.

Bliek, J., Terhal, P., van den Bogaard, M. J., Maas, S., Hamel, B., Salieb-Beugelaar, G., Simon, M., Letteboer, T., van der Smagt, J., Kroes, H. & Mannens, M. (2006). Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype. *Am. J. Hum. Genet.* 78(4), 604-14.

Boonen, S. E., Mackay, D. J., Hahnemann, J. M., Docherty, L., Gronskov, K., Lehmann, A., Larsen, L. G., Haemers, A. P., Kockaerts, Y., Dooms, L., Vu, D. C., Ngoc, C. T., Nguyen, P. B., Kordonouri, O., Sundberg, F., Dayanikli, P., Puthi, V., Acerini, C., Massoud, A. F., Tümer, Z. & Temple, I. K. (2013). Transient neonatal diabetes, ZFP57, and hypomethylation of multiple imprinted loci: a detailed follow-up. *Diabetes Care*. 36(3), 505-12.

Brioude, F., Oliver-Petit, I., Blaise, A., Praz, F., Rossignol, S., Le Jule, M., Thibaud, N., Faussat, A. M., Tauber, M., Le Bouc, Y. & Netchine, I. (2013). CDKN1C mutation affecting the PCNA-binding domain as a cause of familial Russell Silver syndrome. *J. Med. Genet.* 50(12), 823-830.

Brioude, F., Netchine, I. Praz, F., Le Jule, M., Calmel, C., Lacombe, D. Edery, P., Catala, M., Odent, S., Isidor, B., Lyonnet, S., Sigaudy, S., Leheup, B., Audebert-Bellanger, S., Burglen, L., Giuliano, F., Alessandri, J. L., Cormier-Daire, V., Laffargue, F., Blesson, S., Coupier, I., Lespinasse, J., Blanchet, P., Boute, O., Baumann, C., Polak, M., Doray, B., Verloes, A., Viot, G., Le Bouc, Y. & Rossignol, S. (2015). Mutations of the Imprinted CDKN1C Gene as a Cause of the Overgrowth Beckwith-Wiedemann Syndrome: Clinical Spectrum and Functional Characterization. *Hum. Mutat*. 36(9), 894-902.

Bruce, S., Hannula-Jouppi, K., Lindgren, C.M., Lipsanen-Nyman, M. & Kere, J. (2008). Restriction Site-Specific Methylation Studies of Imprinted Genes with Quantitative Real-Time PCR. *Clin. Chem*. 54(3), 491-9.

Bruce, S., Hannula-Jouppi, K., Peltonen, J., Kere, J., Lipsanen-Nyman, M. (2009). Clinically distinct epigenetic subgroups in Silver-Russell syndrome: the degree of H19 hypomethylation associates with phenotype severity and genital and skeletal anomalies. *J. Clin. Endocrinol. Metab.* 94(2), 579-87.

Buiting, K. (2010). Prader-Willi syndrome and Angelman syndrome. *Am. J. Med. Genet. C. Semin. Med. Genet.* 154C(3), 365-76.

Bullman, H., Lever, M. Robinson, D. O., Mackay, D. J., Holder, S. E. & Wakeling, E. L. (2008). Mosaic uniparental disomy of chromosome 11 in patient with Silver-Russell syndrome. *J. Med. Genet*. 45(6), 396-9.

Cattanach, B. M. & Kirk, M. (1985). Differential activity of maternally and paternally derived chromosome regions in mice. *Nature*. 315(6019), 496-8.

Cottereau, E., Mortemousque I, Moizard, M. P. Burglen, L., Lacombe, D., Gilbert-Dussadier, B., Sigaudy, S., Boute, O., David, A., Faivre, L., Amiel, J, Robertson, R., Viana Ramos, F., Bieth, E., Odent, S., Demeer, B., Mathieu, M., Gaillard, D., Van Maldergem, L., Baujat, G., Maystadt, I., Heron, D., Verloes, A., Philip, N., Cormier-Daire, V., Froute, M. F., Pinson, L., Blanchet, P., Sarda, P., Willems, M., Jacquinet, A., Ratbi, I., Van Den Ende, J., Lackmy-Port Lis, M. Goldenberg, A., Bonneau, D., Rossignol, S. & Toutain, A. (2013). Phenotypic spectrum of Simpson-Golabi-Behmel syndrome in a series of 42 cases with a mutation in GPC3 and review of the literature. *Am. J. Med. Genet. C. Semin. Med. Genet.* 163C(2), 92-105.

Crapster, J. A., Hudgins, L., Chen, J. K. & Gomez-Ospina, N. (2017). A novel missense variant in the GLI3 zinc finger domain in a family with digital anomalies. *Am. J. Med. Genet. A*. 173(12), 3221-3225.

Dawson, B. & Trapp, R. G. (2004). *Basic & Clinical Biostatistics*. USA, McGraw-Hill.

DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*. 64(4), 849-59.

de Jong, F., Monuteaux, M. C., van Elburg, R. M., Gillman, M. W. & Belfort, M. B. (2012). Systematic review and meta-analysis of preterm birth and later systolic blood pressure. *Hypertension.* 59(2), 226-234.

Du, P., Kibbe, W. A. & Lin, S. M. lumi: a pipeline for processing Illumina microarray. *Bioinformatics.* 24, 1547-8.

Eggermann, K., Bliek, J., Brioude, F., Algar, E., Buiting, K., Russo, S., Tümer, Z., Monk, D., Moore, G., Antoniadi, T., Macdonald, F., Netchine, I., Lombardi, P., Soenllner, L., Begemann, M., Prawitt, D., Maher, E. R., Mannens, M., Riccio, A., Weksberg, R., Lapunzina, P., Grønskov, K., Mackay, D. J. G. & Eggermann, T. (2016). EMQN best practice guidelines for the molecular genetic testing and reporting of chromosome 11p15 imprinting disorders: Silver-Russell and Beckwith-Wiedemann syndrome. *Eur. J. Hum. Genet*. 24(10), 1377-1387.

Eggermann, T., Algar, E., Lapunzina, P., Mackay, D., Maher, E. R., Mannens, M., Netchine, I., Prawitt, D., Riccio, A., Temple, I. K. & Weksberg, R. (2014a). Clinical utility gene card for: Beckwith-Wiedemann Syndrome. *Eur. J. Hum. Genet*. 22(3).

Eggermann, T., Heilsberg, A. K., Bens, S., Siebert, R., Beygo, J., Buiting, K., Begemann, M. & Soellner, L. (2014b). Additional molecular findings in 11p15-associated imprinting disorders: an urgent need for multi-locus testing. *J. Mol. Med.* 92(7), 769-77.

Eggermann, T., Meyer, E., Obernann, C., Heil, I., Schüler, H., Ranke, M. B., Eggermann, K. & Wollmann, H. A. (2005). Is maternal duplication of 11p15 associated with Silver-Russell syndrome? *J. Med. Genet*. 42(5), e26.

Eggermann, T., Schönherr, N., Jäger, S., Spaich, C., Ranke, M.B., Wollmann, H.A. & Binder, G. (2008). Segmental maternal UPD(7q) in Silver-Russell syndrome. *Clin. Genet.* 74(5), 486-9.

Eggermann, T., Schönherr, N., Meyer, E., Obermann, C., Mavany, M., Eggermann, K., Ranke, M.B. & Wollmann, H.A. (2006). Epigenetic mutations in 11p15 in Silver-Russell syndrome are restricted to the telomeric imprinting domain. *J. Med. Genet.* 43(7), 615-6.

Eggermann, T., Wollmann, H. A., Kuner, R., Eggermann, K., Enders, H., Kaiser, P. & Ranke, M. B. (1997). Molecular studies in 37 Silver-Russell syndrome patients: frequency and etiology of uniparental disomy. *Hum. Genet.* 100(3-4), 415-9.

Ehrich, M., Nelson, M. R., Stanssens, P., Zabeau, M., Liloglou, T., Xinarianos, G., Cantor, C. R., Field, J. K. & van den Boom, D. (2005). Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *PNAS*. 102(44), 15785-15790.

Engel, E. A new genetic concept: Uniparental disomy and its potential effect, isodisomy. (1980). *Am. J. Med. Genet*. 6(2),137-143.

Feinberg, A. P., Ohlsson, R. & Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nat. Rev. Genet*. 7(1), 21-33.

Fisher, A. M., Thomas, N. S., Cockwell, A., Stecko, O., Kerr, B., Temple, I. K. & Clayton, P. (2002). Duplications of chromosome 11p15 of maternal origin result in a phenotype that includes growth retardation. *Hum. Genet*. 111(3), 290-6.

Fokstuen, S. & Kotzot, D. (2014). Chromosomal rearrangements in patients with clinical features of Silver-Russell syndrome. *Am. J. Med. Genet. A*. 164A(6), 1595-605.

Freese, N. H., Norris, D. C. & Loraine A. E. (2016). Integrated Genome Browser: Visual analytics platform for genomics. *Bioinformatics*. 32(14), 2089-95.

Gerbrands, L. C., Haarman, E. G., Hankel, M. A. & Finken, M. J. J. (2017). Cystic fibrosis and Silver-Russell syndrome due to a partial maternal isodisomy of chromosome 7. *Clin. Case Rep.* 5(10), 1697-1700.

Ghasemi, A., Zahediasl, S. (2012). Normality Tests for Statistical Analysis: A Guide for Non-Statisticians. *Int. J. Endocrinol. Metab*. 10(2), 486-489.

Gibson, W. T., Hood, R. L., Zhan, S. H., Bulman, D. E., Fejes, A. P., Moore, R., Mungall, A. J., Eydoux, P., Babul-Hirji, R., An, J., Marra, M. A., FORGE Canada Consortium, Chitayat, D., Boycott, K. M., Weaver, D. D. & Jones, S. J. M. (2012). Mutations in EZH2 Cause Weaver Syndrome. *Am. J. Hum. Genet*. 90(1), 110-118.

Gicquel, C., Rossignol, S., Cabrol, S. Houang, M., Steunou, V., Barbu, V., Danton, F., Thibauld, N., Le Merrer, M., Burgien, L., Bertrand, A.M., Netchine, I. & Le Bouc, Y. (2005). Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat. Genet*. 37(9), 1003-7.

Gustin, R. M., Bichell, T. J., Bubser, M., Daily, J., Filonova, I., Mrelashvili, D., Deutch, A. Y., Colbran, R. J., Weeber, E. J. & Haas, K. F. (2010). Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome. *Neurobiol. Dis*. 39(3), 283-91.

Hannula-Jouppi, K., Muurinen, M., Lipsanen-Nyman, M., Reinius, L. E., Ezer, S., Greco, D. & Kere, J. (2014). Differentially methylated regions in maternal and paternal uniparental disomy for chromosome 7. *Epigenetics.* 9(3), 351-365.

Hannula, K., Kere, J., Pirinen, S., Holmberg, C. & Lipsanen-Nyman, M. (2001b). Do patients with maternal uniparental disomy for chromosome 7 have a distinct mild Silver-Russell phenotype? *J. Med. Genet*. 38(4), 273-278.

Hannula, K., Lipsanen-Nyman, M., Kontiokari, T. & Kere, J. (2001a). A narrow segment of maternal uniparental disomy of chromosome 7q31-qter in Silver-Russell syndrome delimits a candidate gene region. *Am. J. Hum. Genet*. 68(1), 247-253.

Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. (1996). Real time quantitative PCR. *Genome Res*. 6(10), 986-94.

Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E. & Lumey L.H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl. Acad. Sci. USA.* 105(1), 7046-9.

Hitchins, M. P. & Moore, G. E. (2002). Genomic imprinting in fetal growth and development. *Expert Rev. Mol. Med*. 4(11), 1-19.

Hofman, P.L., Regan, F., Jackson, W.E., Jefferies, C., Knight, D.B., Robinson, E.M. & Cutfield, W.S. (2004). Premature birth and later insulin resistance. *N. Engl. J. Med*. 351(21), 2179-2186.

Horan, G. S., Wu, K., Wolgemuth, D. J. & Behringer, R. R. Homeotic transformation of cervical vertebrae in Hoxa-4 mutant mice. (1994). *Proc. Natl. Acad. Sci. USA*. 91(26), 12644-8.

Horsthemke, B. Mechanisms of imprint dysregulation. (2010). *Am. J. Med. Genet. C. Semin. Med. Genet.* 154C(3), 321-8.

Hult, M., Tornhammar, P., Ueda, P., Chima, C., Bonamy, A.K., Ozumba, B. & Norman, M. (2010). Hypertension, diabetes and overweight: looming legacies of the Biafran famine. *PLoS One*. 5(10), e13582.

Huxley, R. R., Shiell, A. W. & Law, C. M. (2000). The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of literature. *J. Hypertens.* 18(7), 815-831.

Höglund, P., Holmberg, C., de la Chapelle, A. & Kere, J. (1994). Paternal isodisomy for chromosome 7 is compatible with normal growth and development in a patient with congenital chloride diarrhea. *Am. J. Hum. Genet*. 55(4), 747-52.

Ishida, M., Monk, D., Duncan, A. J., Abu-Amero, S., Chong, J., Ring, S. M., Pembrey, M. E., Hindmarsh, P. C., Whittaker, J. C., Stanier, P. & Moore, G. E. (2012). Maternal Inheritance of a Promoter Variant in the Imprinted PHLDA2 Gene Significantly Increases Birth Weight. *Am. J. Hum. Genet.* 90(4), 715-719.

Ishida, M. & Moore, G. E. (2013). The role of imprinted genes in humans. *Mol. Aspects Med.* 34(4), 826-40.

Jirtle R.L., Imprinted Gene Databases, www.geneimprint.com, Accessed May 20th 2018.

Johnson, W.E., Li, C. & Rabinovic, A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics.* 8(1), 118-27.

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet*. 13(7), 484-92.

Jones, P. A. & Liang, G. (2009). Rethinking how DNA methylation patterns are maintained. *Nat. Rev. Genet*. 10(11), 805-11.

Joyce, C. A., Sharp, A., Walker, J. M., Bullman, H. & Temple, I. K. (1999). Duplication of 7p12.1-p13, including GRB10 and IGFBP1, in a mother and daughter with features of Silver-Russell syndrome. *Hum. Genet*. 105(3), 273-80.

Kannenberg, K., Urban, C. & Binder, G. (2012). Increased incidence of aberrant DNA methylation within diverse imprinted gene loci outside of IGF2/H19 in Silver-Russell syndrome. *Clin. Genet*. 81(4), 366-77.

Karlberg, N., Jalanko, H., Perheentupa, J. & Lipsanen-Nyman, M. (2004). Mulibrey nanism: clinical features and diagnostic criteria. *J. Med. Genet*. 41(2), 92-8.

Kim, J., Bretz, C. L. & Lee, S. (2015). Epigenetic instability of imprinted genes in human cancers. *Nucleic Acids Res.* 43(22), 10689-10699.

Koerner, M. V., Pauler, F. M., Huang, R. & Barlow, D. P. (2009). The function of non-coding RNAs in genomic imprinting. *Development*. 136(11), 1771-83.

Kostic, D. & Capecchi, M. R. (1994). Targeted disruptions of the murine Hoxa-4 and Hoxa-6 genes result in homeotic transformations of components of the vertebral column. *Mech. Dev*. 46(3), 231-47.

Kotzot, D. (2001). Complex and segmental uniparental disomy (UPD): review and lessons from rare chromosomal complements. *J. Med. Genet*. 38, 497-507.

Kotzot, D. (2008a). Complex and segmental uniparental disomy updated. *J. Med. Genet*. 45(9), 545-56.

Kotzot, D. (2008b) Maternal uniparental disomy 7 and Silver-Russell syndrome – clinical update and comparison with other subgroups. *Eur. J. Med. Genet*. 51(5), 444-51.

Kotzot, D., Schmitt, S., Bernasconi, F., Robinson, W.P., Lurie, I.W., Ilyina, H., Méhes, K., Hamel, B.C., Otten, B.J., Hergersberg, M., Werder, E., Schoenle, E. & Schinzel, A. (1995). Uniparental disomy 7 in Silver-Russell syndrome and primordial growth retardation. *Hum. Mol. Genet*. 4(4), 583-7.

Le Caignec, C., Isidor, B., de Pointbriand, U., David, V., Audrezet, M. P., Ferec, C. & David, A. (2007). Third case of paternal isodisomy for chromosome 7 with cystic fibrosis: a new patient presenting with normal growth. *Am. J. Med. Genet*. 143A(22), 2696-9.

Lenhard, B., Sandelin, A. & Carninci, P. (2012). Metazoan promoters: emerging characteristics and insights into transcriptional regulation. *Nat. Rev. Genet*. 13, 233-245.

Lewis, A. & Reik, W. (2006). How imprinting centres work. *Cytogenet. Genome Res*. 113(1-4), 81-9.

Lewis, E. B. (1992). Clusters of master control genes regulate the development of higher organisms. *JAMA*. 267(11), 1524-1531.

Li, Y., Xie, C., Murphy, S.K., Skaar, D., Nye, M., Vidal, A.C., Cecil, K.M., Dietrich K.N., Puga, A., Jirtle, R.L. & Hoyo, C. (2016). Lead exposure during early human development and DNA methylation of imprinted gene regulatory elements in adulthood. *Environ. Health Perspect*. 124(5), 666-673.

Luedi, P. P., Dietrich, F. S., Weidman, J. R., Bosko, J. M., Jirtle, R. L. & Hartemink, A. J. (2007) Computational and experimental identification of novel human imprinted genes. *Genome Res*. 17(12), 1723-30.

Mackay, D. J. G. & Temple, I. K. (2017). Human imprinting disorders: Principles, practice, problems and progress. *Eur. J. Med. Genet*. 60(11), 618-626.

Maksimovic, J., Gordon, L. & Oshlack, A. (2012). SWAN: Subset-quantile within array normalization for Illumina Infinium HumanMethylation450 BeadChips. *Genome Biol.* 13(6), R44.

Marsaud, C., Rossignol, S., Tounian, P., Netchine, I. & Dubern, B. (2015). Prevalence and management of gastrointestinal manifestations in Silver-Russell syndrome. *Arch. Dis. Child*. 100(4), 353-358.

Matsuzaki, H., Loi, H., Dong, S., Tsai, Y. Y., Fang, J., Law, J., Di, X., Liu, W. M., Yang, G., Liu, G., Huang, J., Kennedy, G. C., Ryder, T. B., Marcus, G. A., Walsh, P. S., Shriver, M. D., Puck, J. M., Jones, K. W. & Mei, R. (2004). Parallel genotyping of over 10,000 SNPs using a one-primer assay on a high-density oligonucleotide array. *Genome Res*. 14(3), 414-25.

McDonald-McGinn, D. M., Sullivan, K. E., Marino, B. Philip, N., Swillen, A., Vorstman, J. A., Zackai, E. H., Emanuel, B. S., Vermeesch, J. R., Morrow, B. E., Scambler, P. J. & Bassett, A. S. (2015). 22q11.2 deletion syndrome. *Nat. Rev. Dis. Primers*. 19(1), 15071.

McGrath, J. & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*. 37(1), 179-83.

Mergenthaler, S., Wollmann, H. A., Burger, B., Eggermann, K., Kaiser, P., Ranke, M. B., Schwanitz, G. & Eggermann, T. Formation of uniparental disomy 7 delineated from new cases and a UPD7 case after trisomy 7 rescue. Presentation of own results and review of the literature. *Ann. Genet*. 43(1), 15-21.

Monk, D., Wakeling, E. L., Proud, V., Hitchins, M., Abu-Amero, S. N., Stanier, P., Preece, M. A. & Moore, G. E. (2000). Duplication of 7p11.2-p13, including GRB10, in Silver-Russell syndrome. *Am. J. Hum. Genet*. 66(1), 36-46.

Moore, G. E., Ishida, M., Demetriou, C., Al-Olabi, L., Leon, L. J., Thomas, A. C., Abu-Amero, S., Frost, J. M., Stafford, J. L., Chaoqun, Y., Duncan, A. J., Baigel, R., Brimioulle, M., Iglesias-Platas, I., Apostolidou, S., Aggarwal, R., Whittaker, J. C., Syngelaki, A., Nicolaides, K. H., Regan, L., Monk, D. & Stanier, P. (2015). The role and interaction of imprinted genes in human fetal growth. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 370(1663), 20140074.

Moore, T. & Haig, D. (1991). Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet*. 7(2), 45-9.

Murphy, S.K., Adigun, A., Huang, Z., Overcash, F., Wang, F., Jirtle, R.L., Schildkraut, J.M., Murtha, A.P., Iversen, E.S. & Hoyo, C. (2012). Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke. *Gene.* 494(1), 36-43.

Murray, P. G., Hanson, D., Coulson, T., Stevens, A., Whatmore, A., Poole, R. L., Mackay, D. J., Black, G. C. & Clayton, P. E. (2013). 3-M syndrome: a growth disorder associated with IGF2 silencing. *Endocr. Connect*. 2(4), 225-35.

Muurinen, M., Hannula-Jouppi, K., Reinius, L. E., Söderhäll, C., Merid, S. K., Bergström, A., Melén, E., Pershagen, G., Lipsanen-Nyman, M., Greco, D. & Kere, J. (2017). Hypomethylation of *HOXA4* promoter is common in Silver-Russell syndrome and associates with stature in healthy children. *Sci. Rep*. 7(1),15693

Nannya, Y., Sanada, M., Nakazaki, K., Hosoya, N., Wang, L., Hangaishi, A., Kurokawa, M., Chiba, S., Bailey, D. K., Kennedy, G. C. & Ogawa, S. (2005). A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* 65, 6071-9.

National Institutes of Health. "Talking Glossary of Genetic Terms." National Human Genome Research Institute. 11 October 2018, from https://www.genome.gov/glossary/

Netchine, I., Rossignol, S., Dufourg, M. N., Azzi, S., Rousseau, A., Perin, L., Houang, M., Steunou, V., Esteva, B., Thibaud, N., Demay, M. C., Danton, F., Petriczko, E., Bertrand, A. M., Heinrichs, C., Carel, J. C., Loeuille, G. A., Pinto, G., Jacquemont, M. L., Gicquel, C., Cabrol, S. & Le Bouc, Y. (2007) 11p15 imprinting center region 1 loss of methylation is a common and specific cause of typical Russell-Silver syndrome: clinical scoring system and epigenetic-phenotypic correlations. *J. Clin. Endocrinol. Metab.* 92(8), 3148-54.

Pan, Y., McCaskill, C. D., Thompson, K. H., Hicks, J., Casey, B., Shaffer, L. G. & Craigen, W. J. (1998). Paternal isodisomy of chromosome 7 associated with complete situs inversus and immotile cilia. *Am. J. Hum. Genet*. 62(6), 1551-5.

Piedrahita, J. A. (2011). The role of imprinted genes in fetal growth abnormalities. *Birth Defects Res. A Clin. Mol. Teratol*. 91(8), 682-92.

Pinborg, A., Loft, A., Romundstad, L. B., Wennerholm, U. B., Söderström-Anttila, V., Bergh, C. & Aittomäki, K. (2016). Epigenetics and assisted reproductive technologies. *Acta Obstet. Gynecol. Scand*. 95(1), 10-5.

Plasschaert, R. N. & Bartolomei, M. S. (2014). Genomic imprinting in development, growth, behavior and stem cells. *Development.* 141(9), 1805-13.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F. T., Douglas, S. H., Field, S. L., Bell, S. M., Combaret, V., Puisieux, A., Mighell, A. J., Robinson, P. A., Inglehearn, C. F., Isaacs, J. D. & Markham, A. F. (2003). Real-time PCR based on SYBR-Green I fluorescence: An alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnol*. 3(18).

Poole, R. L, Docherty, L. E., Al Sayegh, A., Caliebe, A., Turner, C., Baple, E., Wakeling, E., Harrison, L., Lehmann, A., Temple, I. K. & Mackay, D. J. K. (2013). Targeted methylation testing of a patient cohort broadens the epigenetic and clinical description of imprinting disorders. *Am. J. Med. Genet. A*. 161A(9), 2174-2182.

Preece, M. A., Price, S. M., Davies, V., Clough, L., Stanier, P., Trembath, R. C. & Moore, G. E. (1997). Maternal uniparental disomy 7 in Silver-Russell syndrome. *J. Med. Genet.* 34(1), 6-9.

Prickett, A. R., Ishida, M., Böhm, S., Frost, J. M., Puszyk, W., Abu-Amero, S., Stanier, P., Schulz, R., Moore, G. E. & Oakey, R. J. (2015). Genome-Wide Methylation Analysis in Silver-Russell Syndrome Patients. *Hum. Genet*. 134(3), 317-32.

Quinonez, S. C. & Innis, J. W. (2014). Human HOX gene disorders. *Mol. Genet. Metab.* 111(1), 4-15.

Reboul, M. P., Tandonnet, O., Biteau, N., Belet-de Putter, C., Rebouissoux, L., Moradkhani, K., Vu, P. Y., Saura, R., Arveiler, B., Lacombe, D., Taine, L. & Iron, A. (2006). Mosaic maternal uniparental isodisomy for chromosome 7q21-qter. *Clin. Genet*. 70(3), 207-13.

Robertson, K. D. (2005). DNA methylation and human disease. *Nat. Rev. Genet*. 6(8), 597-610.

Robinson, W. P., Lorda-Sanchez, I., Malcolm, S., Langlois, S., Schuffenhauer, S. Knoblauch, H., Horsthemke, B. & Schinzel, A. A. (1993). Increased parental ages and uniparental disomy 15: a paternal age effect? *Eur. J. Hum. Genet*. 1(4), 280-286.

Rochtus, A., Martin-Trujillo, A., Izzi, B., Elli, F., Garin, I., Linglart, A., Mantovani, G., Perez de Nanclares, G., Thiele, S., Decallonne, B., Van Geet, C., Monk, D. & Freson, K. (2016). Genome-wide DNA methylation analysis of pseudohypoparathyroidism patients with GNAS imprinting defects. *Clin. Epigenetics*. 8(10).

Rudaks, L. I., Nicholl J. K., Bratkovic, D. & Barnett, C. P. (2011). Short stature due to 15q26 microdeletion involving IGF1R: Report of an additional case and review of the literature. *Am. J. Med. Genet. A*. 155A(12), 3139-3143.

Russell, A. (1954). A Syndrome of intra-uterine dwarfism recognizable at birth with cranio-facial dysostosis, disproportionately short arms, and other anomalies (5 examples). *Proc. R. Soc. Med.* 47(12), 1040-1044.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. (1988). *Science*. 239(4839), 487-91.

Schinzel, A. A., Robinson, W. P., Binkert, F., Fanconi, A. (1994). An interstitial deletion of proximal 8q (q11-q13) in a girl with Silver-Russell syndrome-like features. *Clin. Dysmorphol.* 3, 63-9.

Schmittgen, T. D. & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* 3(6), 1101-8.

Schneider, A., Hommel, G. & Blettner, M. Linear Regression Analysis: Part 14 of a Series on Evaluation of Scientific Publications. *Dtsch. Arztebl. Int*. 107(44), 776-782.

Schock, G. & Traeger, T. (2011). Techniques to overcome bottlenecks in epigenetics research. https://www.qiagen.com/nl/resources/resourcedetail?id=230b8d92-8c30-4062-b48d-b30affb0caa3&lang=en

Schönherr, N., Meyer, E., Eggermann, K., Ranke, M.B., Wollmann, H.A. & Eggermann, T. (2006). (Epi)mutations in 11p15 significantly contribute to Silver-Russell syndrome: but are they generally involved in growth retardation? *Eur. J. Med. Genet*. 49(5), 414-8.

Shokolenko, I. N. & Alexeyev, M. F. (2015). Mitochondrial DNA: A disposable genome? *Biochim. Biophys. Acta.* 1852(9), 1805-9.

Silver, H. K., Kiyasu, W., George, J. & Dearner, W. C. (1953). Syndrome of congenital hemihypertrophy, shortening of stature and elevated urinary gonadotropins. *Pediatrics.* 12(4), 368-373.

Simpkin, A. J., Suderman, M., Gaunt, T. R., Lyttleton, O., McArdle, W. L., Ring, S. M., Tilling, K., Davey Smith, G. & Relton, C. L. (2015). Longitudinal analysis of DNA methylation associated with birth weight and gestational age. *Hum. Mol. Genet.* 24(13), 3752-63.

Smeets, C. C., Zandwijken, G. R., Renes, J. S. & Hokken-Koelega A. C. (2016). Long-Term Results of GH Treatment in Silver-Russell Syndrome (SRS): Do They Benefit the Same as Non-SRS Short-SGA? *J. Endocrinol. Metab*. 101(5), 2105-12.

Smyth, G. K. Limma: linear models for microarray data. In: Gentleman, R., Carey, V., Dudoit, S., Irizarry, R. & Huber, W. *Bioinformatics and computational Biology Solutions using R and Bioconductor.* New York, Springer, p. 397-420.

Spence, J. E., Perciaccante, R. G., Greig, G. M., Willard, H. F., Ledbetter, D. H., Hejtmancik, J. F., Pollack, M. S., O'Brien, W. E. & Beaudet, A. L. (1988). Uniparental disomy as a mechanism for human genetic disease. *Am. J. Hum. Genet*. 42(2), 217–226.

Spengler, S., Schönherr, N., Binder, G., Wollmann, H. A., Fricke-Otto, S., Mühlenberg, R., Denecke, B., Baudis, M., Eggermann, T. (2010). Submicroscopic chromosomal imbalances in idiopathic Silver-Russell Syndrome (SRS): the SRS phenotype overlaps with the 12q14 microdeletion syndrome. *J. Med. Genet*. 47(5), 356-60.

Spotila, L. D., Sereda, L., & Prockop, D. J. (1992). Partial isodisomy for maternal chromosome 7 and short stature in an individual with a mutation at the COL1A2 locus. *Am. J. Hum. Genet. 51*(6), 1396–1405.

Steenman, M. J., Rainier, S., Dobry, C. J., Grundy, P., Horon, I. L. & Feinberg, A. P. (1994). Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour. *Nat. Genet*. 7(3), 433-9.

Surani, M. A., Barton, S. C. & Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature.* 308(5959), 548-50.

Szathmari, M., Vasarhelyi, B., Reusz, G. & Tulassay, T. (2000). Adult cardiovascular risk factors in premature babies. *Lancet.* 356(9233), 939–940.

Tatton-Brown, K., Pilz, D. T., Orstavik, K. H., Patton, M., Barber, J. C., Collinson, M. N., Maloney, V. K., Huang, S., Crolla, J. A., Marks, K., Ormerod, E., Thompson, P., Nawaz, Z., Lese-Martin, C., Tomkins, S., Waits, P., Rahman, N. & McEntagart, M. (2009). 15q overgrowth syndrome: a newly recognized phenotype associated with overgrowth, learning difficulties, characteristic facial appearance, renal anomalies and increased dosage of distal chromosome 15q. *Am. J. Med. Genet. A*. 149A(2), 147-54.

Ting, J. C., Roberson, E. D., Miller, N. D., Lysholm-Bernacchi, A., Stephan, D. A., Capone, G. T., Ruczinski, I., Thomas, G. H., Pevsner, J. (2007). Visualization of uniparental inheritance, Mendelian inconsistencies, deletions, and parent of origin effects in single nucleotide polymorphism trio data with SNPtrio. *Hum. Mutat.* 28, 1225-35.

Tollefsbol, T. (2011). *Handbook of Epigenetics*. USA, Elsevier. p. 583-5

Turner, C. L. S., Mackay, D. M., Callaway, J. L. A., Docherty, L. E., Poole, R. L., Bullman, H., Lever, M., Castle, B. M., Kivuva, E. C., Turnpenny, P. D., Mehta, S. G., Mansour, S., Wakeling, E. L., Mathew, V., Madden, J., Davies, J. H. & Temple, I. K. (2010). Methylation analysis of 79 patients with growth restriction reveals novel patterns of methylation change at imprinted loci. *Eur. J. Hum. Genet*. 18(6), 648-655.

Voss, R., Ben-Simon, E., Avital, A., Godfrey, S., Zlotogora, J., Dagan, J., Tikochinski, Y. & Hillel, J. (1989). Isodisomy of chromosome 7 in a patient with cystic fibrosis: could uniparental disomy be common in humans? *Am. J. Hum. Genet.* 45(3), 373–380.

Voss, T. C. & Hager, G. L. (2014) Dynamic regulation of transcriptional states by chromatin and transcription factors. *Nat. Rev. Genet*. 15, 69-81.

Wakeling, E. L., Amero, S. A., Alders, M., Bliek, J., Forsythe, E., Kumar, S., Lim, D. H., MacDonald, F., Mackay, D. J., Maher, E. R., Moore, G. E., Poole, R. L., Price, S. M., Tangeraas, T., Turner, C. L., Van Haelst, M. M., Willoughby, C., Temple, I. K. & Cobben, J. M. (2010). Epigenotype-phenotype correlations in Silver-Russell syndrome. *J. Med. Genet.* 47(11), 760-8.

Wakeling, E. L., Brioude, F., Lokulo-Sodipe, O., O'Connell, S. M., Salem, J., Bliek, J., Canton, A. P., Chrzanowska, K. H., Davies, J. H., Dias, R. P., Dubern, B., Elbracht, M., Giabicani, E., Grimberg, A., Grønskov, K., Hokken-Koelega, A. C., Jorge, A. A., Kagami, M., Linglart, A., Maghnie, M., Mohnike, K., Monk, D., Moore, G. E., Murray, P. G., Ogata, T., Petit, I. O., Russo, S., Said, E., Toumba, M., Tümer, Z., Binder, G., Eggermann, T., Harbison, M. D., Temple, I. K., Mackay, D. J. & Netchine, I. (2017). Diagnosis and Management of Silver-Russell Syndrome: First International Consensus Statement. *Nat. Rev. Endocrinol.* 13(2), 105-24.

Walter, J. & Paulsen, M. (2003). Imprinting and disease. *Semin. Cell Dev. Biol*. 14(1), 101-10.

Waterland, R. A. & Jirtle, R. L. (2004). Early nutrition, epigenetic changes and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition.* 20(1), 63-68.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M. & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat. Neurosci*. 7(8), 847-54.

Weksberg, R., Hughes, S., Moldovan, L., Bassett, A. S. Chow, E. W. C. & Squire, J. A. (2005). A method for accurate detection of genomic microdeletions using real-time quantitative PCR. *BMC Genomics*. 6(180).

Willyard, C. (2018). New human gene tally reignites debate. *Nature.* 558(7710), 354-355.

Winchester, L., You, C. & Ragoussis, J. (2009). Comparing CNV detection methods for SNP arrays. *Brief. Funct. Genomic. Proteomic*. 8(5), 353-366.

Wollmann, H. A., Kirchner, T., Enders, H., Preece, M. A. & Ranke, M. B. (1995). Growth and symptoms in Silver-Russell syndrome: review on the basis of 386 patients. *Eur. J. Pediatr.* 154(12), 958–968.

APPENDIX: ORIGINAL PUBLICATIONS